Some Studies on Graminicolous *Didymella* spp. in New Zealand

A Thesis
submitted in partial fulfilment
of the requirements for the degree
of

Doctor of Philosophy

at the

University Of Canterbury

by

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1999

To my family and Nathalie

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LIST OF ABBREVIATIONS

ANOVA =	Analysis of Variance
g =	grams
h =	hour
l =	litre
min =	minute
µl =	microlitre
ml =	millilitre
µm =	micrometer
mm =	millimetre
PDA =	Potato dextrose agar
spp. =	species
UV =	ultraviolet light

ACKNOWLEDGMENTS

The people I would like to thank first and foremost are my family and here they are in no particular order. My husband Hamish Cochrane for trying to keep me focused and for tolerating late night panic sessions, listening to my drivel about *Didymella* and reading drafts.

My daughter Alexandra for providing much needed hindrance and making sure I never got enough sleep.

My mother and my father for their constant drive to get the thing finished. My Mother for her ability to listen, counsel and for help in the field. My sister Vicky, for her help in the field. My Father for his ability to sound like a cracked record and I quote "Finish your PhD."

Thank you to all of you for putting up with me during the long long years that it has taken to complete this thesis and be assured I never want to repeat the experience.

I would like to thank my Associate Supervisor, Dr Matthew Cromey (Crop and Food Research) for all his help and guidance over the course of this thesis. Thank you for organising field trials, employing me for periods over the summer season and for reading the final draft over Christmas. I would also like to thank Matthew for the dimensions of the graminicolous *Didymella* spp. which he collected during the 1992/93 field season. I would also like to thank my supervisor Associate Professor A.L.J. Cole for his help and guidance over the course of this thesis. I would also like to thank him for giving up part of the Christmas break to read the final draft.

I would also like to thank various people at the University of Canterbury, especially Manfred Ingerfeld for his help with the transmission electron microscopy and calming influence. Megan Mahan, Jackie Healy and Dr John Klena for help with molecular work. Craig Galliee for his help in the Micro lab.

For help with field work: Alison Seyb, Hamish Cochrane, Barbara Mace, and Vicky Mace.

The people I would like to thank at Crop and Food Research are:
Helen, Suvi, and Heather for providing light relief and guidance; Stan Ebdon for the help in the glasshouse; and Dr John Marshall and Simon Bulman for incredible help with the molecular work when it all seemed hopeless.

For reading my drafts: Barbara Mace, Hamish Cochrane and Ted Mace

And finally but certainly not least, I would also like to thank Messers Bassett, Mulholland, Goodwin and Irwin (1995/96) and Messers Brand, Marrs, Lill and Brooker for providing land and management for the trials and everyone who sowed and harvested the trials. I would also like to thank the University of Canterbury for the Doctoral Scholarship.

ABSTRACT

The aim of this thesis was to elucidate aspects of the biology of *Didymella* species present on Graminicolous hosts in New Zealand. The taxonomy of *Didymella* spp. and their anamorphs was investigated. Four main types were found and these corresponded to *Didymella exitialis D. phleina, D.graminicola and Didymosphaeria loliina*. The anamorph states were identified as the *Ascochyta* state of *D. exitialis*, *Ascochyta phyllachoroides* Sacc. and Malb. Forma *melicae*, *Ascochyta* state of *D. graminicola* and the *Ascochyta* state of *D. loliina*. Two of the isolates identified as the *Ascochyta* state of *D. graminicola* could also be classified as *A. sorghi*.

The production of the teleomorph in culture was not achieved on a consistent basis. The teleomorph was produced by four isolates that were plated on CD-V_8 agar. Only pseudothecia and ascii were produced; no ascospores could be found. The teleomorph was produced on one other occasion, when isolates on PDA had been dried down slowly.

Conidia of *Didymella* germinate on leaf surfaces between three and five hours after inoculation at 25^0C and 98% RH. Production of appressoria occurs approximately 30 hours after inoculation at 20^0C and 98% RH. The method of penetration of the leaf surface is direct with no preferred sites for penetration. A subcuticular, intramural mycelium then develops between the cuticle and the epidermis until the host becomes stressed, or the leaf material starts to senesce. Symptoms affecting less than one percent of the leaf area developed on inoculated plants, no increase in the rate of leaf senescence of infected plants compared to uninoculated plants was observed.

Didymella spp. are frequently isolated from symptomless surface sterilised leaf material during the wheat-growing season. *Didymella* spp. are initially isolated after the first leaf unfolds and can be isolated sporadically from other leaves throughout the growing season, until the leaves senesce. During the 1995/96 growing season the application of azoxystrobin significantly reduced the levels of *Didymella* spp. isolated from leaves and the amount of sporulation on leaf material. In 1996/97 azoxystrobin had

no significant effect upon the level of *Didymella* spp. cultured from leaf material, however, the level of sporulation on leaf material was less than those leaves treated with tebuconazole or a water control. The levels of *Didymella* spp., bacteria and *Sporobolomyces* spp. cultured from leaf tissue were not significantly correlated with weather conditions.

Fungicide sprays did not affect the level of *Sporobolomyces* on leaf tissue. In field surveys *Didymella* spp. were the most common fungi on senesced leaf tissue. The levels of *Septoria nodorum* and *Septoria tritici* were found to be very low in both the 1995/96 and 1996/97 field surveys. No cultivars resistant to *Didymella* spp. were identified.

1.0 INTRODUCTION

A fungus causing a leaf scorch of wheat was discovered during 1988 in Canterbury and Southland and identified *Didymella exitialis* (Morini) Müller. *Didymella* spp. are Loculascomycetes and their anamorphs are in the genus *Ascochyta*. *Didymella* is an enigma in both its identity and its role in causing leaf scorch of wheat and barley. The identity of the species present in New Zealand remain unknown as the dimensions of the perithecia, ascii and ascospores for the teleomorph state and the dimensions of the pycnidia and conidia for the anamorph state do not match the published descriptions of Punithalingham (1979a) (Mace unpublished). In New Zealand it is an abundant fungus on senesced leaves of wheat and barley but its effect on crop yield remains unknown (Cromey *et al.* 1994a).

Didymella exitialis (Morini) Müller, and *Didymella phleina* Punithalingham and Årsvoll have both been recorded as being associated with leaf scorch symptoms on wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare*). *Didymella exitialis* has also been implicated as an aeroallergen and as causing premature senescence of wheat leaves. *D. exitialis* is a prevalent fungus on wheat and barley crops around the world and its perithecia and pycnidia are often present in abundant quantities on senescing or senescent leaf tissue (Punithalingham 1979a). It is suggested to be either an endophyte or weakly pathogenic on wheat (Frankland and Gregory 1973). The mode of transmission of *D. exitialis* remains unknown, however it is presumed to be via airborne ascospores and conidia (Punithalingham 1979a). The seasonal persistence of *D. exitialis* remains obscure and little is known of the epidemiology of leaf scorch (Punithalingham 1979a).

1.01 Hosts

Graminicolous *Ascochyta* species have been recorded on most grasses. In Canterbury the grasses present at the margins of wheat and barley crops are often infected and these may act as overwintering sources or as reservoirs of inoculum. Wheat and barley are affected, according to the literature by two different species, *Didymella exitialis* and *Didymella phleina* respectively. New Zealand had in 1995, 52 000 ha in grain crops and 1295 farms which were predominantly cropping farms (Ministry of

Agriculture and Fisheries (MAF) 1998). World - wide the production of wheat exceeded 600 million tonnes for the 1997/98 season, and New Zealand imported 216,000 tonnes (MAF 1998).

1.02 Taxonomy of *Didymella*

The taxonomic distinction between the anamorph states of both teleomorphs *Didymella exitialis* and *Didymella phleina* appears to be indistinct because of their similarities to other *Ascochyta* species. The anamorph of *D. exitialis* is known as the *Ascochyta* state and can appear similar to the *Ascochyta* state of *Didymella graminicola* (Punithalingham 1979b). Ascospores of *Didymella exitialis* can also morphologically resemble the ascospores of *Didymella autumnalis,* however these two states can be distinguished by the slightly different morphology of the anamorph states (Punithalingham 1979b).

Ascochyta phyllachoroides Sacc. and Malb. forma *melicae* Fautrey is the anamorph of *Didymella phleina* and in culture is very difficult to distinguish from *Ascochyta avenae* (Petrak) Sprague and Johnson. The problems encountered are due to the variation of conidial sizes between strains and within the same strain, after repeated subculturing (Riesen 1987).

The production of the teleomorph of both *Didymella* spp. in culture would help to resolve taxonomic problems, however culture conditions have not been optimised for *D. exitialis*. Pseudothecia of *D. phleina* have been produced in culture after 35 days in malt extract broth on wheat straw (Riesen 1987), however, there appear to be no reports pseudothecia of *D. exitialis* being produced in culture.

1.021 Appearance of *D. exitialis* on host substrate and in culture.

Didymella exitialis appears on its host as dark brown to black subglobose pseudothecia that are immersed in wheat or barley leaves (Punithalingham 1979a). Pseudothecia or pycnidia may be produced within scorch lesions. The scorch lesions have a tan centre clearly delimited by a dark brown border (Mace 1994). The asci are cylindrical to subclavate or broadly obovoid, arranged in a relatively flat layer and

contain eight ascospores. The ascus wall is bitunicate and thick and the ascospores are hyaline and medianly uniseptate with a slight constriction at the septum (Punithalingham 1979a).

The *Ascochyta* anamorph is readily produced in culture. Cultures on potato dextrose agar appear floccose with buff or white blotches and the reverse is fuscous black (Punithalingham 1979b). Pycnidia are formed sparsely and are yellowish brown to greyish sepia in colour. The conidia are hyaline, uniseptate, not constricted at the septum and guttulate. They are also rounded at the base and the apex, and can be cylindrical to oblong or broadly ellipsoid. The *Ascochyta* state of *D. exitialis* resembles *Ascochyta hordeicola* and the *Ascochyta* state of *D. graminicola* because of the variability of pycnidial size and conidial size, shape and colouration (Punithalingham 1979a).

Didymella phleina has yellowish brown to chestnut brown pseudothecia (Punithalingham 1979b). The asci are straight, cylindrical to subclavate and arranged in an almost flat layer. The size of the ascus is larger than that of *D. exitialis*. The ascospores are medianly uniseptate and are fusiform to ellipsoid in shape with acute ends. The pycnidia are yellowish brown in colour. The conidia are straw yellow in colour, the apex is rounded and the base is either flat or rounded. They are oblong to broadly ellipsoid in shape and can have smooth to very rough conidial walls (Punithalingham 1979b).

1.022 Disease impact on wheat and endophytic nature of *Didymella exitialis*

In the United Kingdom the disease caused by *Didymella exitialis* is known as *Didymella* leaf scorch and it is considered to be one of the major causes of late season loss of leaf material (Punithalingham 1979a). The fungus has been recorded on wheat and barley in Switzerland, Britain, Africa and Asia (Punithalingham 1974a).

D. exitialis was found to be the most abundant ascomycetous endophyte in green wheat leaves in Switzerland (Riesen 1985 and Sieber 1985 in Riesen 1987). In further

research in Switzerland, *D. exitialis* was isolated from 3.1% of wheat culms, roots and leaves (Sieber *et al.* 1988).

Apart from *D. exitialis*, *D. phleina* is the only *Didymella* spp. that has been isolated from cereal leaves (Årsvoll 1975 in Riesen 1987). *D. phleina* has also been recorded as a pathogen of *Lolium* spp., *Phleum pratense* L. and other grasses but only under favourable conditions for infection of the hosts (Årsvoll 1975 in Riesen 1987), although the favourable conditions were not defined.

Endophytes were first described as "curious outgrowths" (uredospores, teleutospores) from wheat leaves called "exanthemata" (Unger 1833 in Tarr 1972). Leveille (1846 in Riesen 1987) recognised that these outgrowths were fungal structures and introduced the term "endophytic fungi". Today this term describes any fungus existing within a host plant without causing visible symptoms. Endophytic fungi occur in all plant families recorded so far (Carroll and Carroll 1978, Petrini and Carroll 1981 in Fisher and Anson 1986, Petrini and Dreyfuss 1981 in Petrini and Fisher 1986).

There are many different definitions for endophytic fungi. They are, according to the definition of symbiosis in the 'Dictionary of the Fungi' (Hawkesworth *et al.* 1983), "symbionts occurring in living plant tissue." The actual term 'symbiont' can itself cause confusion as it is presumed by some that symbiosis is an association between two organisms of different species from which both partners benefit, however it is normally accepted as referring to two organisms of different species living together, not necessarily to their mutual benefit but not to their detriment (Lawrence 1989). Another definition of endophyte is that used by Sieber *et al.* (1988) who state that "any fungus isolated from the inside of the plant tissue is an endophyte." The latter definition covers any plant pathogenic fungus that is latent in the host however when the latent period is over the endophytic relationship ceases.

There are no hard and fast rules; a fungus may exist in an endophytic way during a latent period and then become pathogenic, or alternatively a fungus may be an endophyte unless the host is somehow weakened at which time it may parasitise the

host. The relation to the host may be mutualistic as in mycorrhizas and *Neotyphodium* endophytes of forage grasses, antagonistic (pathogenic) or neutral as in *Cladosporium* spp. on wheat (Cook 1977).

Endophytes are generally Ascomycetes (Petrini and Fisher 1986). Endophytic fungi colonise stems and leaves of plants without visible symptoms and are often very closely related to virulent pathogens. They may protect the host against infection by plant pathogens, however, due to the evidence that they are directly evolved from plant pathogens they may cause pathogenic symptoms when the host is stressed (Miller 1981, Andrews *et al.* 1980). The distinction between endophytes and latent pathogens becomes blurred in agricultural situations (Nathaniels and Taylor 1983, Kulik 1984) as pathogens may exist endophytically in the weed species growing in the same field (Hepperely *et al.* 1985 in Carroll 1988). Current agricultural practices may in fact encourage latent fungi to be become pathogenic by disturbing the natural equilibrium in the phyllosphere (Carroll 1988).

1.03 *Didymella* species in the Canterbury and Southland regions.

During November 1988, Drs. I.C. Harvey and F.R. Sanderson found members of the genus *Didymella* on wheat in the Southland and Canterbury regions (Dr I.C. Harvey pers. comm., 1994). *Didymella* spp. were found to be present on many leaves infected with *Septoria nodorum*. Isolates obtained from Southland and Canterbury appeared to be distinct, although this may have reflected regional variation within a single species.

During a field survey of wheat crops during the 1990/91 growing season *D. exitialis* was identified on nine cultivars and in 19 of the 24 crops surveyed. The pseudothecia were seen in late November and as the crops senesced they became more common (Cromey *et al.* 1994a). Pseudothecia were found abundantly over the entire leaf surface and were often associated with leaf scorching symptoms. Lesions were elongate in shape with diffuse margins and were tan to dark brown in colour. Only the *Ascochyta* anamorph was produced in culture.

In a study in 1993, the *Ascochyta* anamorph of *D. exitialis* was first isolated from surface sterilised green symptomless flag leaves of wheat at growth stage (GS) 61 (Zadoks *et al.* 1974) (beginning of anthesis) (Cromey *et al.*, 1994b). Isolation of *D. exitialis* increased until there was no green tissue present for isolation due to senescence. A field survey of 58 wheat crops (GS 83-87) in Canterbury showed that *D. exitialis* was the most common pathogen on wheat leaves, present in all crops surveyed, with an average of 25 % leaf area affected (Cromey *et al.* 1994b). Disease incidence of *D. exitialis* was low during the 1994/95 growing season, due to the drier November to January period than that experienced the previous year. Despite this, *Didymella* and its anamorph were the most common pathogens present on wheat flag leaves that were collected during a field survey of 61 Canterbury wheat crops (Cromey and Mace 1995).

D. phleina, which is the only other *Didymella* sp. recorded on cereal leaves, was recorded in New Zealand from symptomless green barley tissue by Riesen (1987). Barley plants inoculated with a spore suspension of *Ascochyta phyllachoroides* f. *melicae* (the anamorph of *D. phleina*) showed no disease symptoms, however, the fungus was shown to be present in 14 of the 40 leaf lamina samples from the inoculated plants indicating its endophytic nature (Riesen 1987).

D. phleina was found to be a common endophyte by Riesen and Close (1987) as it was isolated from 20% of field grown barley leaves, however, it appeared to exist as an endophyte whose presence was transient, depending on the presence of external inoculum and climatic conditions.

When yields of fungicide-sprayed and unsprayed plots of field grown barley were compared it was found that the plots sprayed with propiconazole had higher yields (Riesen and Close 1987). They suggested that in the sprayed plots either cytokinin properties of the fungicide or control of *D. phleina* contributed to the increased yield.

1.04 Life cycle

The life cycle of *Didymella* spp., on graminicolous hosts is largely unknown (Punithalingham 1979a). Transmission appears to be via airborne ascospores, which are abundant in the air above wheat and barley crops (Frankland and Gregory 1973).

1.05 The infection process

The results of plant inoculation studies with *D. exitialis* under greenhouse conditions by various researchers have proved to be equivocal. When pycnidiospore suspensions of *D. exitialis,* were sprayed onto wheat plants, leaf infection was detectable only microscopically, as a slight halo (Ahrens and Schöpfer 1983). It was therefore suggested that *D. exitialis* was either a secondary invader or a weak parasite. Pseudothecia were only formed after three - four weeks under near - UV light. Cromey *et al.* (1994a) inoculated wheat plants with a pycndiospore suspension. Symptom development occurred after two weeks on the margins and the tips of leaves. *D. exitialis* was reisolated from the leaves and pseudothecia developed on inoculated leaves approximately four weeks later. In a similar study Mace (1994) showed wheat plant symptoms of leaf scorch which only affected 1% or less of the leaf area of the host plants tested. It was suggested therefore by Cromey *et al.* (1994b) that the fungus had a long period of latency before symptom development. *Didymella* enters its host directly and establishes itself in a subcuticular intramural location. Pseudothecia of *Didymella* are formed as the leaf tissue becomes senescent (Mace 1994).

1.06 Control

The control of *Didymella* spp. has not been well studied. Paul (1983) indicated yield losses could be as high as 15% between susceptible and tolerant cultivars; what he meant by susceptible and tolerant species is not clear, but it is likely that tolerant indicates a cultivar which is infected by *Didymella,* but exhibits no yield effects. Desmel (propiconazol) and ortho-difolatan were trialed by Paul (1983) but neither achieved control.

It is not known what effect *Didymella* has on yield in New Zealand or what fungicides control *Didymella. Didymella* leaf scorch is often confused with *Septoria*

glume blotch in the field due to the similarities in lesion appearance and so the effect of fungicide application remains unknown. The fungicides chlorothalonil and tebuconazole were trialed during the 1994/1995 in three trials in Canterbury, however, because of a dry November to January period little or no yield differences between treated and untreated plots were found (Cromey and Mace 1995). *Didymella* was only isolated at three of the 13 sampling times and from a maximum of 20% of leaves (Cromey and Mace 1995).

1.07 Resistance

Paul (1983) examined forty-eight winter wheat cultivars for their reactions to natural *Didymella* infections. The severity of leaf scorch varied between cultivars but all were more or less susceptible. A difference in the leaf area covered with pseudothecia was recorded between cultivars in the field trials measured by Cromey *et al.* (1994a). 'Otane' and 'Norseman' were severely affected while 'Monad' and 'Kokako' were mainly unaffected (Cromey *et al.* 1994a).

1.08 The role of *D. exitialis* in late summer asthma

Fungal spores have a widely recognised role in the occurrence of late summer asthma (Frankland and Gregory 1973). The ascospores of *D. exitialis* have been shown to be present in airspora over barley fields in England, reaching concentrations of up to 10^{5}-10^{6} per m^3 of air (Frankland and Gregory 1973). Ascospores of *D. exitialis* have been identified by Corbaz (1969) over wheat fields in Switzerland and Last (1955b) recorded ascospores similar to those of *D. exitialis* present in the airspora over mildew infected crops.

Ascospores of *D. exitialis* have been implicated in late summer asthma (Frankland and Gregory 1973, Harries *et al.* 1985, Allitt 1986, von Wahl and Kersten 1991). The problem, in studying the role of the spores of *Didymella exitialis* in an allergenic response, is the lack of well-defined extracts for the pharmaceutical industry (von Wahl and Kersten 1991). This requires the sporulation of *D. exitialis* under laboratory conditions and culture conditions still have not been optimised to produce the teleomorph in vitro.

In Derby, England, acreages of wheat and barley have more than doubled in the 10 years from 1981 to 1991 (Corden and Millington 1994). As this increase has occurred, an upward trend in the number of *Didymella* ascospores present in the airspora has also occurred. The ascospores are present in maximum concentrations during the wheat-growing season from June to September. No link in the rate of spore discharge with rainfall has been identified. The greatest concentrations of *Didymella* ascospores were found during cereal harvest (Jackson 1984 in Corden and Millington 1994, Harries *et al.* 1985). Packe and Ayres (1985) and Wahl and Kersten (1991) found that the release of ascospores was not temperature-dependent, but high temperatures before rainfall increased the quantity of emitted ascospores.

The initial allergenic reaction in humans to *Didymella* ascospores is the production of IgE antibodies. The isolates of *Didymella* ascospores used in the study carried out by Harries *et al.* (1985) into the allergenic effects of *Didymella* ascospores were relatively unpurified as they came from spore traps. *Alternaria alternata* and *Cladosporium herbarum* also stimulate these antibodies, this makes it difficult to establish the role of *Didymella,* however, the symptoms of late summer asthma first appeared before the first recorded rise of *Alternaria* spores but coincided with the first increase of *Didymella* spores. Some of the individuals tested reacted to the *Didymella* extracts only, but the role of *Didymella* ascospores cannot be confirmed until extracts suitable for skin prick tests are available (Harries *et al.* 1995).

A study into the presence of hyaline one septate (HIS) ascospores in airspora was carried out by Allitt (1986). The HIS ascospores isolated from air were cultured and were found to be members of 6 taxa of *Ascochyta.* These were the *Ascochyta* states of *Didymella exitialis*, *D. phleina, A. hordei, A. hordei var europa* and *A. leptospora.*

A heterogeneous range of HIS ascospores including at least 6 taxa was identified. It is possible that only common taxa were found as relatively few ascospores were isolated. Late colouration and roughening of the ascospores was observed which has not been reported before (Allitt 1986) and thus this may have implications for the

taxonomy of isolates. Late changes in the ascospore wall could indicate changes as to the allergenic components present, which in turn could partially provide an explanation for the different responses of patients to the extracts used for allergenic testing (Allitt 1986).

1.09 Aims and objectives of the study

1/ To investigate any differential resistance to *Didymella* spp. by New Zealand wheat cultivars.

2/ To determine the yield reduction caused by natural *Didymella* spp. infections in field trials.

3/ To investigate the use of chemicals to control *Didymella* leaf scorch and 'latent' infection.

4/ To record the frequency of fungal diseases on wheat both in field trials and field surveys encompassing most of the cereal growing areas of New Zealand

5/ To investigate the presence and time of entry of *Didymella* spp. into a crop.

6/ To investigate the infection processes of *Didymella* spp. under different conditions of temperature and humidity using light and transmission electron microscopy.

7/ To define the conditions required to produce the teleomorph of *D. exitialis* in culture.

8/ To identify and compare graminicolous *Didymella* spp. in New Zealand using traditional morphological and molecular taxonomic methods.

2.0 FIELD INVESTIGATIONS OF *DIDYMELLA* SPECIES

2.01 Introduction

It is difficult to eradicate *Didymella* from a wheat crop and therefore its influence on crop yield remains elusive. *Didymella* is the most common fungus found sporulating on senesced wheat leaves and it is often isolated from symptomless green leaf tissue (Cromey *et al.* 1994a, 1994b, Cromey and Mace 1995). The fungus remains latent until flag leaf senescence occurs; at which point the fungus colonises the leaves (Cromey *et al.* 1994a). As has been mentioned previously there are no fungicides known that control *Didymella,* nor are there any resistant cultivars. If little or no reduction in yield is found in New Zealand then it begs the question is *Didymella* in fact an endophyte or is it a weak latent pathogen?

2.02 Resistance

Cromey *et al.* (1994a) found large differences in the leaf areas affected in different cultivars. They found that Otane and Norseman were affected the greatest whilst Monad and Kokako were less affected. However, Cromey (pers. comm.) suggested that this may have been due to the different developmental stages that the crops were at when sampled.

2.03 Fungicides

Two fungicides were used in this study. The first was tebuconazole, which is a triazole fungicide. The mode of action of triazoles such as tebuconazole is the inhibition of sterol biosynthesis in both the host and the fungus. This affects the defence reaction of the host making it less susceptible to infection (Smolka and Wolf 1983). The second fungicide used in this study was azoxystrobin which it is a broad spectrum fungicide that inhibits mitochondrial respiration (it has the same mode of action as naturally occurring strobilurins) (Godwin *et al.* 1994).

2.04 Endophytes

Generally speaking endophytes are ascomycetes with few exceptions and are found amongst Loculoascomycetes, Discomycetes and Pyrenomycetes (Petrini 1986 in Carroll and Carroll 1988). In endophyte surveys certain fungal genera are reported regardless of the host plant sampled. In some situations the endophyte may be found in 100% of the host population but usually are found in a smaller percentage of host plants (Carroll and Carroll 1978 in Carroll 1988, Petrini 1986 in Carroll 1988).

"Generality of endophytic mutualism" (Carroll 1988)

"1/ Large numbers of host plants over a wide geographic range contain the endophyte which causes minimal symptoms.
2/ Transmission of the fungus must occur via horizontal and vertical means.
3/ The fungus must be present in all host tissue and must disperse many infectious units, however, if the fungus is only present in one particular organ then the infection rate of that organ must be high.
4/ Toxic secondary metabolites are produced.
5/ The fungus must be taxonomically related to other known herbivore and pathogen antagonists (for example *Neotyphodium, Phomopsis* or *Lophodermium*)."

It has been suggested by Fisher and Anson. (1986) that senescence and death of older photosynthetic organs may be hastened by endophytes. Also suggested from available evidence is that endophytes have evolved directly from plant pathogens and thus may cause pathogenic symptoms when the host is compromised in some way (Miller 1981, Andrews *et al.* 1985 in Carroll 1988).

2.05 *Sporobolomyces*

It was observed during the 1995/96 wheat growing season that levels of *Didymella* sporulating were higher on tebuconazole treated leaves than the control and the azoxystrobin treated leaves. It was suggested by Cromey (pers. comm.) that perhaps the phylloplane population of a species that was antagonistic to *Didymella* was reduced

or eradicated by the tebuconazole. Due to the important role that *Sporobolomyces* has in the phyllosphere, the effects of fungicide treatment on this species were investigated.

Di Menna (1959) found that in New Zealand, *Rhodotorula* spp. and *Sporobolomyces* were the most frequent members of the Cryptococcaceae and Sporobolomycetaceae respectively, on ryegrass and clover leaves with as many as $3.2x10^7$ cells being detected per gram fresh weight of leaf. The rate difference at which populations of *Sporobolomyces* and bacteria increase possibly indicates differences in the nutrient amount and type at different stages of plant growth, however, this cannot be a generalisation as some fungi can be isolated from very young tissue (Hudson and Webster 1958). It has also been found that there are greater numbers of *Sporobolomyces* isolated from leaves that have been treated with fertiliser, compared with leaves that have not (Last 1955b). The actual distribution of *Sporobolomyces* on the upper and lower surfaces of leaves is variable, however, with more found on the tips of leaves (Last and Warren 1972).

2.06 Latent infections

The term latency, when applied to phytopathogenic fungi, describes the period between penetration and the appearance of symptoms in their life cycle (Gäumann 1951 in Verhoeff 1974). For latent infection to exist the following conditions must be met:
1/ No macroscopic symptoms are evident, nor does mycelium proliferate during a dormant phase just after penetration of the host.
2/ Ultimately a parasitic relationship between the fungus and host must occur (Verhoeff 1974).

The definition provided by the Federation of British Plant Pathologists (Cook *et al.* 1981) in 1973 for a parasitic relationship is "... an organism exists in an intimate association with a living organism from which it derives an essential part of the material for its existence while conferring no benefit in return."

Didymella may not be a latent pathogen as defined by Verhoeff (1974): it depends on whether it changes from its quiescent to active state in either living or dead host tissue. If it does so in the living tissue it is a parasitic fungus.

2.07 The confusion between the distinction of a latent and an endophytic nature

The above definitions of latency and endophytes are relatively rigid and do not take in to account the vagaries of a biological system. Somewhat looser definitions do exist, for example that of Sieber *et al.* (1988). The key difference between the two definitions is that a latent infection eventually causes disease symptoms. It is not known whether, under certain environmental conditions, endophytes may also cause disease or may stress the plant, thus allowing a pathogen to take hold.

Many studies have been carried out into the isolation and identification of bacteria and fungi from symptomless host tissue. From these studies, it has been found that the presence of various micro-organisms differs according to the age of the host tissue, the cultivar, and the prevailing environmental conditions. One such study was that carried out by Dixit and Gupta (1981) in which 33 species of fungi and two bacterial species were isolated from young and mature leaves. Other studies have indicated that the most dominant saprophytic phyllosphere fungi are *Cladosporium* spp., *Aureobasidium pullulans* (de Bary) Arnaud, "pink" yeast (mainly *Sporobolomyces* spp.) and white yeasts (mainly *Cryptococcus*) (Fokkema 1971, Fokkema and Van der Meulen 1976 in Bashi and Fokkema 1977). The above fungi have been found to antagonise *Cochliobolus sativus* Ito and Kuribayashi, on rye (Fokkema 1973) and *Septoria nodorum* (berk.) Berk. on wheat (Fokkema and Van der Meulen 1976 in Bashi and Fokkema 1976).

Micro-organisms that colonise aerial plant parts without any visible pathogenic effect are called phyllosphere saprophytes (Potter 1910 in Last 1955a, Kerling 1958 in Cabral 1985, Ruinen 1961, Last and Deighton 1965,). Many researchers have found that a positive yield response is obtained after a fungicide application in the absence of any

disease symptoms in cereal crops (Dickinson 1973, Dickinson and Walpole 1975, Fehrmann *et al.* 1978, Priestley 1981 and Priestley and Bayles 1982). Green leaf area is retained for a longer period and senescence is delayed in fungicide treated plots (Jenkins *et al.* 1972, Mappes and Hampel 1977, and Davies *et al.* 1984). It is speculated that these differences may be caused by a reduction in the numbers of phyllosphere micro-organisms (Dickinson 1973, Jenkyn and Prew 1973, Smedgaard-Petersen 1986).

Ruinen (1966) and MacNamara and Dickinson (1981) suggested that *Sporobolomyces* and *Cryptococcus* spp. may be harmful to plants by causing cuticle degradation. Heightened defence reactions to endophytes or phyllosphere organisms is also unaccounted for and may stress a plant so that it is susceptible to the proliferation of latent or saprophytic fungi (MacNamara and Dickinson 1981). Promotion of senescence in mature cereal leaves may be caused by filamentous saprophytic fungi (Tolstrup 1984 in Jachmann and Fehrmann 1989, Tolstrup and Smedgaard Petersen 1984 in Jachmann and Fehrmann 1989).

2.08 Yield, endophytes, environment, fungicides and disease

The application of fungicides to cereals has become standard practice in modern agriculture. Apart from the control of pathogens, it is established that these chemicals may inhibit the growth of saprophytic and weakly parasitic fungi on the aerial parts of plants (Dickinson and Walpole 1975).

Yield responses in seven winter wheat cultivars were related to the amount of green leaf area that remained after anthesis and this was increased by the application of fungicides (Priestly 1981). Disease reduction played a major role in the increase in yield. Yield was more closely correlated with green leaf area than disease reduction. Either the fungicides have a direct physiological effect on the host, or the control of saprophytes increases yield. The fungicides may also control undetected diseases.

Environmental conditions play a major role in the disease reactions of cultivars (Fehrmann *et al.* 1978). In the same paper research by Bauers (1977) and Hanuss and

Prillwitz (1977 pers. comm. in Fehrmann *et al.*) was discussed. An increase in yield was obtained after the application of an MBC fungicide or captafol in the absence of any eyespot disease pressure. On average, a yield increase of 2-3% was obtained over and above that due to the control of *Pseudocerosporella herpotrichoides.* The extra cost of spraying was covered even in the absence of disease. The increase in yield cannot be due to a direct effect on the host metabolism as captafol is non-systemic.

Other research has confirmed findings of increased yield in crops treated with fungicides in the absence of disease pressure (Jachmann and Fehrmann 1989). They inoculated spring wheat in the glasshouse with one of the following phyllosphere inhabitants *Cladosporium herbarum*, *C. cladosporioides*, *Aureobasidium pullulans*, *Epicoccum purpurascens*, *Alternaria alternata*, or white yeasts. Accelerated senescence and yellowing was shown in the plants inoculated with either of the two *Cladosporium* spp. This seems to indicate that under certain conditions *Cladosporium* spp. can influence senescence, thus explaining some unexpected increases in yield following fungicide application. The other phyllosphere organisms did not appear to affect senescence.

2.09 Aims

1/ To investigate the microbial ecology of wheat leaves. In 1995/96 leaf positions 1,5, and flag and during the 1996/97 field season, all leaf positions with a special emphasis on the flag leaves (this will include any culturable pathogens present in their latent phases).

2/ To establish the effects of fungicides on the microbial ecology of the flag leaves of wheat using the same methods as above but investigating the flag leaves only.

3/ To establish when *Didymella* first enters the crop at each leaf position and when sporulation is observed. The differences in the fluctuations of the fungus over time with respect to the prevailing environmental conditions will be investigated.

4/ To establish which diseases are present in the field trial and the percentage of the leaf area affected

5/ To obtain as many isolates of *Didymella* as possible from all the wheat growing areas of New Zealand, and barley and oat crops. Grasses such as cocksfoot, Yorkshire fog, prairie and rye will also be examined and isolates obtained where possible.

2.10 Materials and methods

2.101 Trials and assessments

2.1011 1995/96

Four field trials were sown in Mid-Canterbury within commercial spring wheat crops at Chertsey (36 plots), Norwood (36 plots), Mitcham (12 plots) and Methven (12 plots). The wheat cv. Endeavour was grown at Chertsey and Mitcham and cv. Monad was grown in the other two trials. Plots were 10m x 1.5m in each trial. The trials received the same agronomic treatment as the surrounding crops where normal commercial agronomic practice was followed, except that no fungicides apart from treatment fungicides were applied .

Fungicide treatments (187.5 gai/ha tebuconazole as Folicur, azoxystrobin at 250gai/ha, or a water control) were applied at GS 47, 59, 65 (one timing per plot), in the large trials and at GS59 in the smaller trials. Each treatment was replicated four times in a complete randomised block design. Fungicides were applied using a compressed air back-pack sprayer with a hand-held boom fitted with Tee-jet nozzles (Spraying Systems Co no. 25 core, D2 discs) applying 300 litres/ha at 300kPa. Control plots were sprayed with water. The treatments were 1, 4, and 7; untreated control GS 47, 59 and 65 respectively, treatments 2, 5, 8; tebuconazole applied at GS 47, 59 and 65 respectively and treatments 3, 6 and 9; azoxystrobin applied at GS 47, 59 and 65 respectively

Plots were harvested when ripe. The harvested grain was weighed and the harvest weight was adjusted to 14% moisture content. Results were analysed using ANOVA.

2.1012 1996/97

Four trials were sown in Mid- Canterbury during 1996. There were 24 plots at each site and the plots were 10m x 1.5m in size. The cultivar used was Monad. One spring wheat trial was sited at Pendarvis, and the other at Methven. The autumn sown crops were sited at Aylesbury and at Highbank. The spraying regime changed from the previous year in that crops were sprayed at boot and mid flowering only. Chemicals were applied as for the previous year. Control plots were left unsprayed. Treatments 1 and 4 were the untreated controls, treatments 2 and 5 had tebuconazole applied at GS 49 and GS 65 respectively and treatments 3 and 6 had azoxystrobin applied at GS 49 and GS 65 respectively. The sampling dates were (1) 23/9/96, (2) 7/10/96, (3) 22/10/96, (4) 5/11/96, (5) 19/11/96, (6) 2/12/96, (7) 16/12/96, (8) 2/1/97 and (9) 22/1/97.

2.1013 1997/98

One field trial of autumn sown wheat cv. Monad was located at Hilton near Geraldine. The spraying regime and plot size was the same as that used in the previous year.

2.102 Assessments of disease, plant pathogens and senescence in the trials

2.1021 1995/96

Trials were assessed weekly and then every two days as crops senesced. Ten - 20 plants/plot depending on the variability of disease at each leaf position were visually assessed using standard area diagrams (James 1971) for leaf area affected by blotch (this assessment encompassed glume blotch caused by *Septoria nodorum* (Berk.) Berk., leaf scorch, causal agent *Didymella* spp. and other leaf scorching, for instance due to herbicide damage, *Puccinia striiformis* Westend. (stripe rust), *Erysiphe graminis* DC. Ex Mèrat (powdery mildew), *Puccinia recondita* Rob. (leaf rust) and senescence. The average value per plot was calculated for each disease present at each leaf position. The rate of senescence was compared between treatments by way of a repeated measures ANOVA. The sampling dates were (1) 21/9/95, (2) 1/10/95, (3) 11/10/95, (4) 22/10/95,

(5) 12/11/95, (6) 20/11/95, (7) 26/11/95, (8) 30/11/95, (9) 4/12/95, (10) 11/12/95, (11) 18/12/95, (12) 2/1/96 and (13) 8/1/96.

Ten flag leaves per plot were collected per plot three weeks after the crops had senesced and examined under a stereomicroscope to determine the percentage leaf area covered by fungal pathogens such as *S. nodorum*, *S. tritici*, *Didymella* spp., *P. recondita, P. striiformis*, *Fusarium* spp. and *Dreschslera* spp. Results were analysed using ANOVA.

2.1022 1996/97

Trials were examined every two weeks until the flag leaf emerged then the crops were examined every five days decreasing to two days as the crops senesced. The assessment was the same as that used during the previous field season.

All trials were scored as to disease development. Leaf material was only collected from the two spring wheat trials. The two winter wheat trials were only sampled 10 days after each fungicide application. The presence of plant pathogens was assessed for all trials after flag leaf senescence using the method in **5.1021**

2.1023 1997/98

No assessments were carried out in this trial.

2.103 Isolations from surface sterilised leaves

2.1031 1995/96 and 1996/97

Ten flag leaves per plot were collected as each crop assessment and were surface sterilised with 0.6% sodium hypochlorite and sterile deionised water for two minutes. After two minutes, the leaves were washed with sterile deionised water. Leaves were then cut into 10 one cm^2 segments which were then plated onto PDA (Gibco) and cultured at room temperature. Any fungi isolated were subcultured on to PDA and identified where possible.

Five flag leaves/plot were collected 10 days after each fungicide application in each trial. Leaves were surface sterilised with 0.6% sodium hypochlorite and sterile deionised water for 2 min. Leaf pieces were then plated on to PDA and cultured at room temperature. Any fungi isolated were subcultured on PDA and identified where possible.

2.104 Enumeration of leaf surface microflora

2.1041 1995/96 and 1996/97

Twenty flag leaves per treatment were collected at each assessment date and cut into 2 cm^2 pieces. Necrotic leaf tips were discarded to avoid over-estimation of the *Cladosporium* population as these flourish on the leaf tips. These were placed in sterile 1% Difco mineral peptone and deionised water in a conical flask. The flasks were shaken for 10 min on a reciprocating shaker operating at 160-170 cycles per min followed by serial dilutions of the leaf washings to 10^0 - 10^{-6}. One ml aliquots of each dilution were then removed from each flask and placed in six sterile Petri dishes per plot. Nutrient agar (NA) and potato dextrose agar (PDA) held at approximately 50^0C were then added to the petri dishes (three dishes each) and the dishes swirled in a figure eight to ensure even distribution of washing fluids. Plates were left to set before being incubated at 20^0C.

The plates were incubated for three days, after which time the predominant colonies were identified where possible and the numbers of bacteria, yeasts and fungi were recorded. Predominant bacterial colonies were isolated to pure culture on nutrient agar and then stored in cryotubes in 20% glycerol and 80% nutrient broth. Yeast cultures were stored in the same manner except in potato dextrose broth (Difco PDB) instead of nutrient broth. Fungi were isolated to pure culture and areas of actively growing mycelia were removed using a no. 2 cork borer. Eight blocks of mycelia were placed in cryotubes with 20% glycerol and stored at -80^0C.

Ten leaf pieces per treatment were collected at each assessment date and were cut in half and then placed in Petri dishes in either the abaxial orientation (five leaves)

or the adaxial orientation (five leaves) according to standard leaf area diagrams. Fifteen millilitres of either molten potato dextrose agar or nutrient agar was added to each dish (three replicates of each). The plates were then left to set and then incubated at 20^0C. The plates were incubated for three days after which time the predominant colonies were described and the numbers of bacteria, yeasts and fungi were recorded. Predominate bacterial types were isolated to pure culture on nutrient agar and gram stained. Fungi were isolated to pure culture and identified where possible. Ten leaves per sample

2.1042 1996/97

One week after each fungicide application, 20 flag leaves per treatment were collected from each of the three trials; Aylesbury, Pendarvis and Methven and subsequent sampling was continued every 14 days until flag leaf senescence. Ten flag leaves were used for leaf washings and the other 10 were used for leaf plating. After incubation at 18-20^0C for three days, population densities were calculated for each saprophyte per unit leaf area.

The remaining methodology follows that of the previous field season apart from the use of leaf clearing and the use of Martin's medium (Appendix 2) instead of PDA for leaf washings and the investigation of *Sporobolomyces* populations on leaf surfaces.

Wheat cv. Monad seeds were sterilised in 100% ethanol for 1 h and then washed 2x in sterile distilled water. The seed coat was removed using sterile tweezers and a scalpel and placed on PDA plates. The grain was then cut into pieces and these were placed on PDA as were entire wheat grains. A range of 10 isolates only were then plated singly on the agar plates and the seeds placed around them. Seeds that had mycelium growing over them at the end of the experiment were planted out in nine cm pots.

The predominant bacterial, yeast and fungal cultures were plated on either PDA or nutrient agar along with representative *Didymella* samples. Isolates were placed either as a streak for bacterial and yeast cultures or as plug (2mm) for fungal cultures 3.5 cm

apart on PDA or nutrient agar in four replicate 9cm petri dishes. Single or dual inoculated plates of the same fungus formed the controls. Inhibition of growth of either isolate was recorded every 48 h using the five modes described by Porter 1924 (in Skidmore and Dickinson 1976) and measurements of colony diameter were also recorded.

These five modes are:

1/ both fungi growing into one another with no visible signs of interaction.

2a/ The fungus under investigation is observed overgrowing the other fungus either above or below the original colony of the second fungus

2b/ The fungus under investigation has been overgrown by the other fungus

3/ A demarcation line of approximately 1-2mm has formed between both fungi as they have almost contacted.

4/ Inhibition of both fungi with a demarcation line of >2mm

A 1-4 scale was used to describe interacting fungi for each mode of colony growth. The intermediate values were more difficult to assign. Interactions were evaluated by assessing the ability of each fungus to inhibit the growth of another fungus.

2.105 Direct counts of micro-organisms present in the phylloplane

Ten flag leaves per treatment were cut into segments and were cleared of chlorophyll using the method of Wolf and Fric (1983) (Chapter 3). Cleared leaves were transferred to plates containing glycerol which were sealed and stored in the dark. Leaves were then placed on microscope slides in a mounting mixture of glycerol and lactic acid (75% and 25% respectively) and were then examined under 40x on an Olympus compound microscope. Fungal and yeast cells numbers were recorded and calculated as the number of spores per mm^2 area of leaf tissue.

2.106 High humidity and leaf imprinting

Five leaves per leaf position were placed on moistened filter paper in plastic Petri dishes and placed under near- UV light (12 hr cycle) at 18^0C. The leaves were examined under a stereo microscope and any fungi present identified where possible.

Leaf imprinting was carried out by pressing the adaxial or abaxial surface of six leaves per sample on to the surface of Martin's medium. The plates were then incubated at 18^0C and examined three days after inoculation and any fungi identified where possible.

2.107 Field survey

2.1071 1995/96 and 1996/97

Commercial cereal crops (barley, wheat, oats, and triticale) and some grasses in mid Canterbury were surveyed during late January 1996 as the crops approached maturity. Twenty flag leaves were collected at random along a diagonal from each crop. The leaves were examined at x25 under an Olympus stereomicroscope and the percentage area affected by various diseases was recorded.

Isolations of *Didymella* were then made from these leaves. This was achieved by removing a small area of leaf tissue containing between two and six perithecia. A small dot of Vaseline was placed on the lid of a water agar plate and the leaf tissue was placed on this with the perithecia held over the agar plate. The lid was then replaced on the plate and the plate was then place on a 45° to the horizontal. The plates were examined after eight hours for the discharged ascospores on the plate. The ascospores were removed singly from the plate using a sterile scalpel and placed on a PDA plate. The plate was then incubated at 18-20^0C and the identity of the isolate as *Ascochyta* spp. was confirmed. The cultures were then used for taxonomic work in the Chapter 5.

2.1072 1996/97

Commercial cereal crops (barley, wheat, oats, and triticale) and the some of the grasses present at the crop margins in the lower North Island, Mid Canterbury, South Canterbury and Southland were surveyed during late January 1997 as the crops approached maturity. The remaining methodology follows that used in 1995/96.

2.108 The effects of fungicide applications on the *Sporobolomyces* populations

2.1081 1996/97 and 1997/98

The method used to isolate *Sporobolomyces* from treated flag leaves was that used by Last (1955). Leaf material came from the Methven and Pendarvis sites during 1996/97 and from the Hilton site during the 1997/98 field seasons. Four flag leaves were collected from each treatment at both of the spring wheat trials. These leaves were then laid in an abaxial or an adaxial orientation (2 replicates of each) across the lower half of a Petri dish (but not touching the media) containing either PDA or Martins medium. The lid was then replaced thus supporting the leaf blade. The plates were left to incubate at 18-20^{0}C for 24 hours. Before the leaves were removed at the end of the incubation period, the area exposed over the medium was calculated by multiplying the mean width of the leaf by the length of the leaf. The plates were then incubated in an inverted position at 18-20^{0}C for 24 hours and then the number of colonies of *Sporobolomyces* were calculated. The results were analysed using ANOVA.

2.109 Resistance

2.1091 1995/96 and 1996/97

Ten flag leaves per plot were collected from the Arable Cultivar Evaluation (ACE) trials in Mid Canterbury during 1995-97. In 1995/96, 17 cultivars were evaluated and 14 in 1996/97. The ACE trials are of a randomised block design, replicated four times and the plot size is 1.2m X 10m. Commercial wheat crops surround the trials and agronomic management was as for the surrounding crop. Two spring wheat sites were chosen per year. During the 1995-96 growing season, the sites were Methven and Cherstey. During 1996-97, the sites were Pendarvis and Methven. Plots were scored for four weeks prior to flag leaf senescence for the percentage senescence and then flag leaves were harvested from all plots at the same stage of senescence. The flag leaves were collected two weeks after each plot had senesced and stored in paper bags until examination.

Leaves were soaked in water in a beaker before being examined under an Olympus stereomicroscope at x25. The percentage leaf area covered by fungal plant pathogens, for example, *S. nodorum, S. tritici, Didymella* spp. and *P. recondita* was assessed for each leaf and the identity of the fungus was confirmed using an Olympus compound microscope. Only three replicates per cultivar were examined and the results were analysed by ANOVA.

2.110 Fungicide experiment

Two fungicides, tebuconazole (Bayer) (187.5gai/ha) and azoxystrobin (Zeneca) (250gai/ha) were investigated to see what effect they had on *Didymella* in culture. Three different concentrations of each fungicide were used. These were ½ field rate (0.125gai/ml of medium for azoxystrobin and 0.09gai/ml of medium for tebuconazole), field rate (0.25gai/ml of medium for azoxystrobin and 0.19gai/ml of medium for tebuconazole) and 2x field rate (0.5gai/ml of medium for azoxystrobin and 0.38gai/ml of medium for tebuconazole),. PDA ($^1/_5$ strength) was made up in 15 ml lots to which the fungicide at the appropriate dilution in sterile distilled water was added. PDA plates with no fungicide were also poured. The fungicide amended PDA was then left to set.

Four *Didymella* isolates were chosen at random from cultures obtained during the 1996/97 field season (Appendix 5) and there were three replicates per isolate per treatment. A no. 4 cork borer was used to cut a disc of mycelium from the margin of an actively growing colony. These were then placed in the centre of each of the plates and the plates were incubated at 20^0C. Colony diameter was measured at on two axes right angles 24, 48, and 72 hours after plating and the growth rate per day was calculated. Results were analysed using a repeated measure ANOVA. After 72 hours the plates were placed under near - UV light to observe whether sporulation of the fungus occurred.

Sixteen day-old cultures of *Didymella* isolated from wheat were used to prepare a spore suspension. The cultures were flooded with sterile deionised water containing 500µg/ml of 'Tween' 20 and the agar surface was then scraped with a sterile microscope

slide. The suspensions were then filtered through sterile muslin and the resulting suspensions were adjusted to approximately 50,000 spores/ml using a haemocytometer.

Slides were prepared with a thin layer of PDA over the surface amended with the different concentrations of fungicides. The control slides had no fungicide present. Twenty µl of spore suspension was then distributed evenly over the agar surface. The slides were left in the laminar flow to dry and then placed in sterile petri dishes and incubated for 24 hour at 20^0C with a 12 h photoperiod. The slides were then examined under x100 and the germination rate of the spores was recorded in 10 fields chosen at random. Results were analysed using a repeated measures ANOVA.

2.20 Results

2.201 Disease

2.2011 1995\96

There were low disease levels in the trials, mainly powdery mildew (*Erysiphe graminis* D.C.) and stripe rust (*Puccinia striiformis* West.). Blotch or leaf scorching was observed infrequently and when it did occur pycnidia of *S. nodorum* and the pseudothecia of *Didymella* were often present in and around the scorch lesions. On 8/1/96, at the Methven trial, there was significantly more blotch ($P<0.05$) on the flag leaves in untreated control plots than in those treated with azoxystrobin.

2.2012 1996\97

Aylesbury

Leaf rust was the most common pathogen in this trial affecting on average 5% of the leaf area of all leaves at sampling date 11/1/97. Up until this date the leaf rust score had consistently been one percent. On the 11/1/97 1% powdery mildew was identified only in the later azoxystrobin treatment.

Methven

This trial had less than one percent incidence leaf rust present up until the 31/12/96 when this figure rose to an average of 54% of leaves, with one percent of the leaf area affected. However, there were no statistical differences between the leaf rust severity in the different treatments.

Pendarvis

This trial also had leaf rust present once again with no real differences between treatments in severity. On the 8/1/97 8% of leaves were affected with an average of 1% of the leaf area; by the 17/1/97 this figure had risen to 9% of leaves affected with 6% of the leaf area. This trial also had scorch symptoms present on the first four leaves; possibly due to herbicide damage. Less than 1% of the leaf area was affected, however, *Didymella* sporulated within these lesions.

Highbank

In the Highbank trial leaf rust was also the most common disease affecting 90% of leaves examined and 4% of the leaf area. Powdery mildew was identified at a level of less than 1% in the third and fourth samples.

2.202 Isolations from surface sterilised leaves

2.2021 Levels of *Didymella* spp., *Sporobolomyces* spp. and bacterial spp. cultured from wheat leaves from the trials carried out during 1995/96 and 1996/97

2.20211 1995/96

Didymella was not cultured and was thus assumed not to be present in any of the trials on 21/09/95 (Tables 2.2, 2.4, 2.6 and 2.7). By the second sample on 1/10/95 *Didymella* was present in all trials in leaf one (Figures 2.2, 2.3, 2.4). Generally *Didymella* entered leaf positions five and eight before the leaves completely unfolded. *Didymella* levels within the trials fluctuated throughout the growing season until the plants senesced (Figures 2.2, 2.3, 2.4,). Few known pathogens were isolated from leaf tissue and the levels of these pathogens also fluctuated throughout the growing season (Table 2.2, 2.4, 2.6 and 2.7). Bacterial levels increased with increasing plant age. The levels of other fungal species, for example *Alternaria* spp., and *Sporobolomyces* also fluctuated over the field season. *Didymella* levels from the trials were correlated using a Spearman rank correlation with the weather data obtained from the NIWA sites at Lincoln, Darfield and Ashburton Township (Figure 2.6, 2.7 and 2.8). As can be seen from these correlations there was no significant correlation between rainfall or temperature and *Didymella* levels.

2.20212 1996/97

Didymella was isolated from the first leaf in the Methven trial at the second sample on 7/10/96 but was not isolated again from this leaf position (Table 2.12 and Figure 2.1). It was never isolated from leaf two. It was isolated from leaf four before it was isolated from leaf three. In the other leaf positions, the levels fluctuated during the growing

season. *Didymella* was isolated from leaf one in the Pendarvis trial on 22/10/96 (Table 2.11 and Figure 2.5). Levels of *Didymella* tended to increase until 16/12/98 when *Didymella* levels reduced from 100% (16/12/96) to 20% in leaf 7 (12/1/97). Bacterial levels increased as leaves became senescent. *Helminthosporium* spp., *Fusarium* spp. and *S. nodorum* were present infrequently and only in low numbers. The levels of the other species isolated also fluctuated throughout the growing season (Tables 2.11 and 2.12 and Figures 2.1 and 2.5). *Didymella*, *Sporobolomyces* spp. and bacterial spp. levels were not correlated with weather data obtained from NIWA sites at Ashburton Township, Darfield and Lincoln during 1996/97 growing season. As can be seen by figures 2.6, 2.7 and 2.8 the levels of the three taxa were not highly correlated with the weather data showing there was no significant effect of temperature and rainfall.

2.2022 Levels of *Didymella* isolated from tebuconazole and azoxystrobin treated leaves.

2.20221 1995/96

In the Methven trial levels of *Didymella* were highest in those leaves treated with tebuconazole (Table 2.3). In the other trials *Didymella* levels were generally highest in the untreated controls, closely followed by the levels present in those leaves treated with tebuconazole (Table 2.3, 2.5, 2.8 and 2.9). The timing of fungicide applications made no significant difference to the levels of *Didymella* present. *Didymella* was not isolated from leaves treated with azoxystrobin in any trial. *Alternaria* spp. levels remained high in all treatments and for all timings of fungicide application.

2.20222 1996/97

Aylesbury (Table 2.10)

Didymella was not isolated from any treatment until 16/12/96, ten days after fungicide application. The flag leaves from the second timing of fungicide applications were only

sampled once and the control and the tebuconazole levels had *Didymella* levels of 100% and 90% respectively. There was no *Didymella* in the azoxystrobin treated leaves. The levels of all other species fluctuated within all treatments and timings of fungicide application.

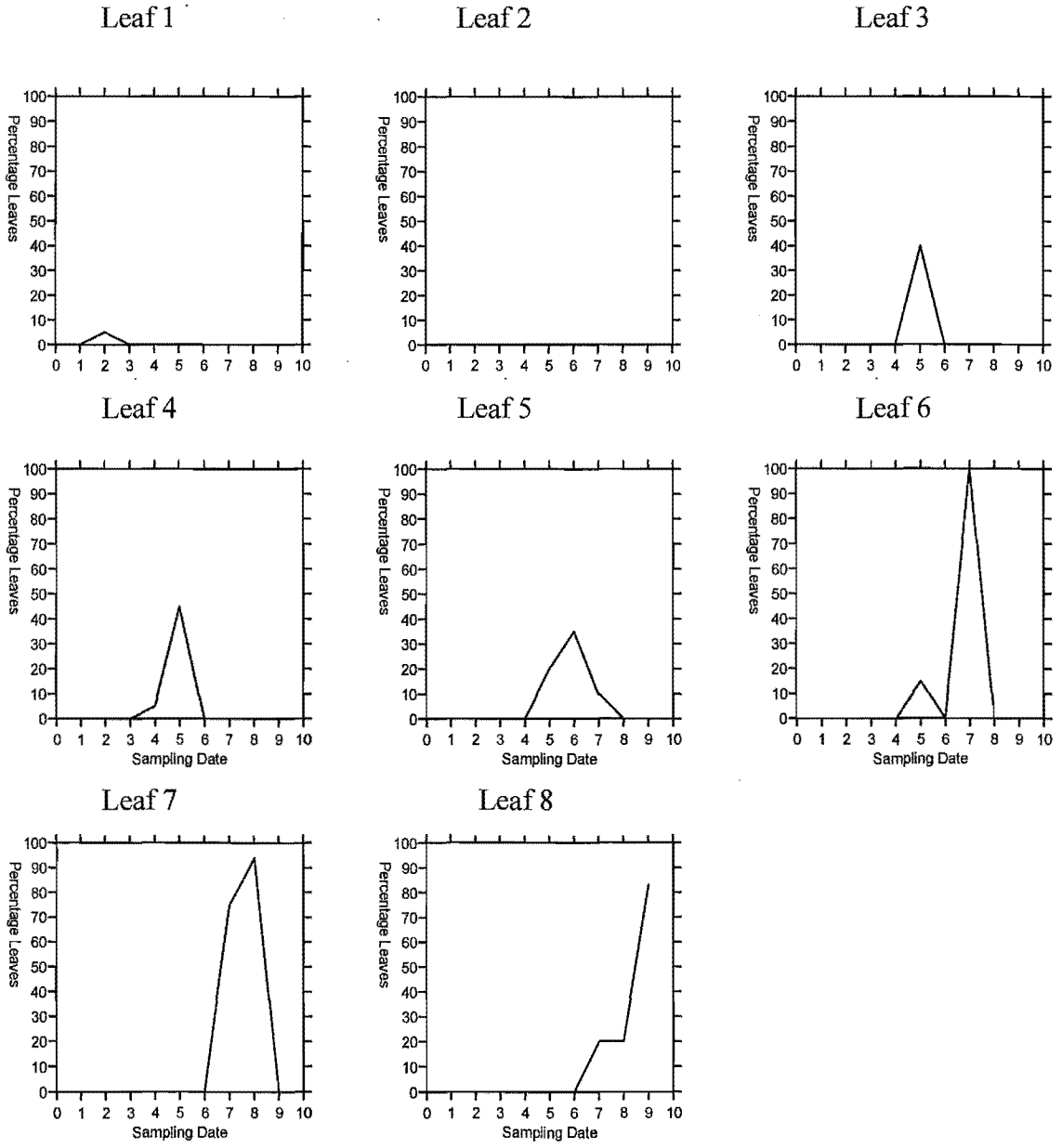

Figure 2.1 The percentage of surface sterilised wheat leaves (leaf positions 1-8) from which *Didymella spp.* were cultured from at the Methven trial 1996/97. The sampling dates were (1) 23/9/96, (2) 7/10/96, (3) 22/10/96, (4) 5/11/96, (5) 19/11/96, (6) 2/12/96, (7) 16/12/96, (8) 2/1/97 and (9) 22/1/97.

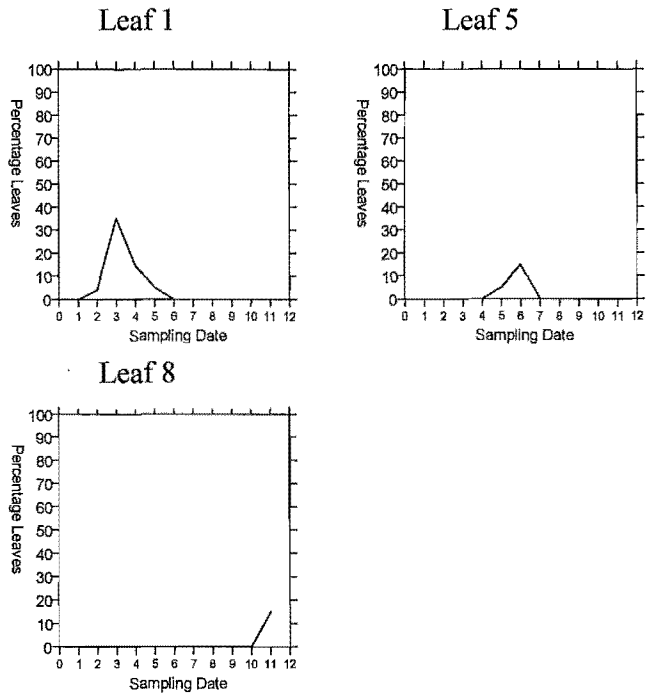

Figure 2.2 The percentage of surface sterilised wheat leaves (leaf positions 1,5,8) from which *Didymella spp.* were cultured from at the Methven trial 1995/96. The sampling dates were (1) 21/9/95, (2) 1/10/95, (3) 11/10/95, (4) 22/10/95, (5) 22/11/95, (6) 20/11/95, (7) 26/11/95, (8) 30/11/95, (9) 4/12/95, (10) 11/12/95, (11) 18/12/95, (12) 2/1/96 and (13) 8/1/96.

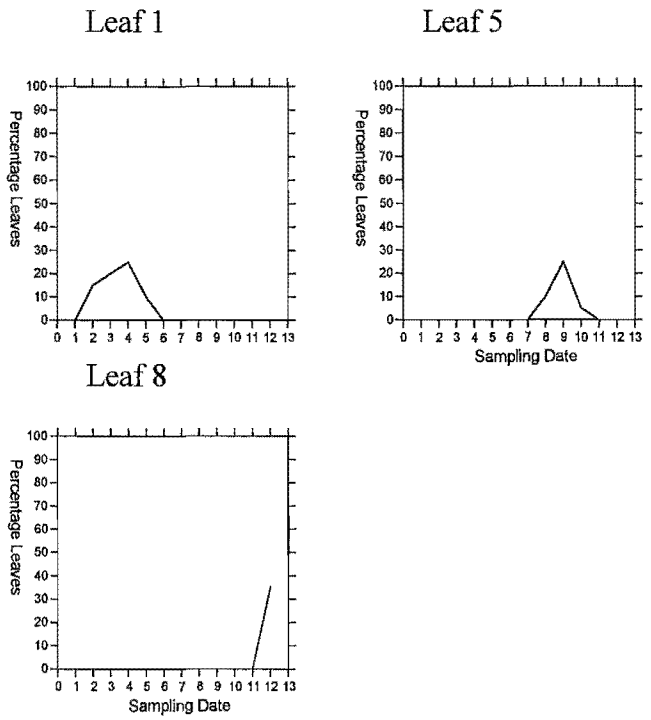

Figure 2.3 The percentage of surface sterilised wheat leaves (leaf positions 1,5,8) from which *Didymella spp.* were cultured from at the Norwood trial 1995/96. The sampling dates were (1) 21/9/95, (2) 1/10/95, (3) 11/10/95, (4) 22/10/95, (5) 22/11/95, (6) 20/11/95, (7) 26/11/95, (8) 30/11/95, (9) 4/12/95, (10) 11/12/95, (11) 18/12/95, (12) 2/1/96 and (13) 8/1/96.

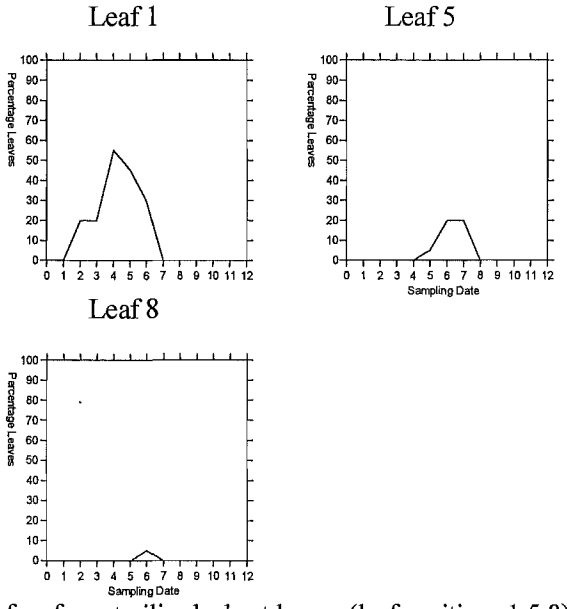

Figure 2.4 The percentage of surface sterilised wheat leaves (leaf positions 1,5,8) from which *Didymella spp.* were cultured from at the Mitcham trial 1995/96. The sampling dates were (1) 21/9/95, (2) 1/10/95, (3) 11/10/95, (4) 22/10/95, (5) 22/11/95, (6) 20/11/95, (7) 26/11/95, (8) 30/11/95, (9) 4/12/95, (10) 11/12/95, (11) 18/12/95, (12) 2/1/96 and (13) 8/1/96.

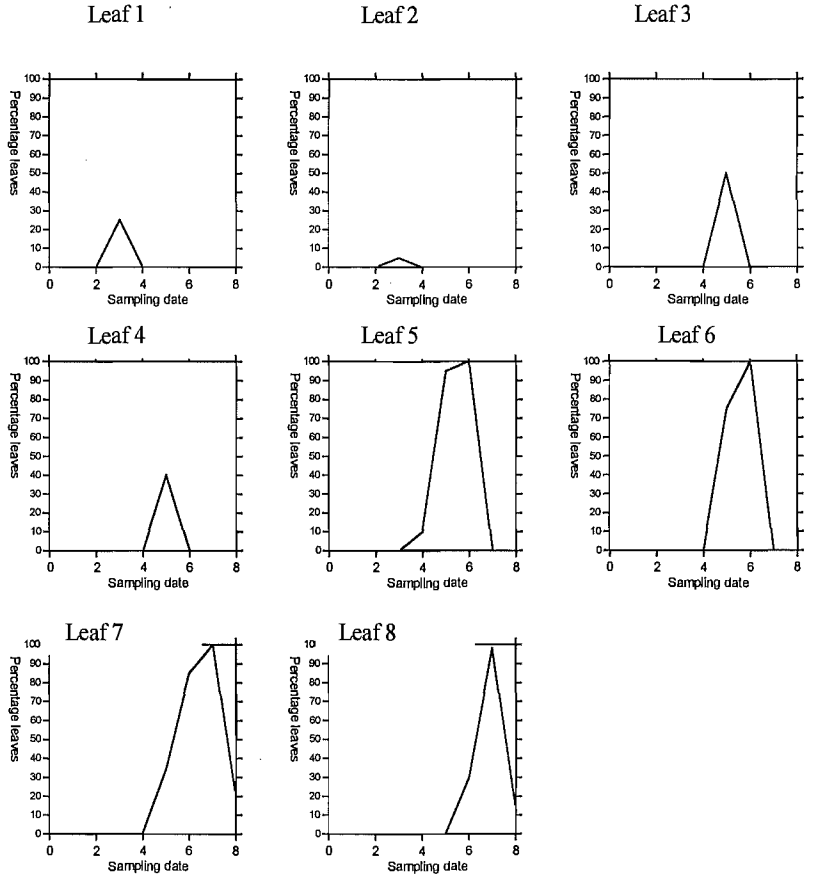

Figure 2.5 The percentage of surface sterilised wheat leaves (leaf positions 1-8) from which *Didymella spp.* were cultured from at the Pendarvis trial 1996/97. The sampling dates were (1) 23/9/96, (2) 7/10/96, (3) 22/10/96, (4) 5/11/96, (5) 19/11/96, (6) 2/12/96, (7) 16/12/96, (8) 2/1/97 and (9) 22/1/97.

Pendarvis (Table 2.14)

Didymella was present in all treatments but not in the 2/12/96 sample for the tebuconazole and the azoxystrobin treated leaves. Levels of other species fluctuated throughout the sampling. Table 2.1 shows that treatment and trial had no effect on the levels of *Didymella*, *Sporobolomyces* and bacteria cultured from green leaf tissue in either the Methven or the Pendarvis trial.

Methven (Table 2.13)

Didymella was present in all treatments apart from the 2/1/97 sample of treatment six (azoxystrobin applied at GS 65). There were no statistically significant differences between *Didymella* levels in any of the treatments. Levels of the other species investigated fluctuated during the sampling period regardless of treatment.

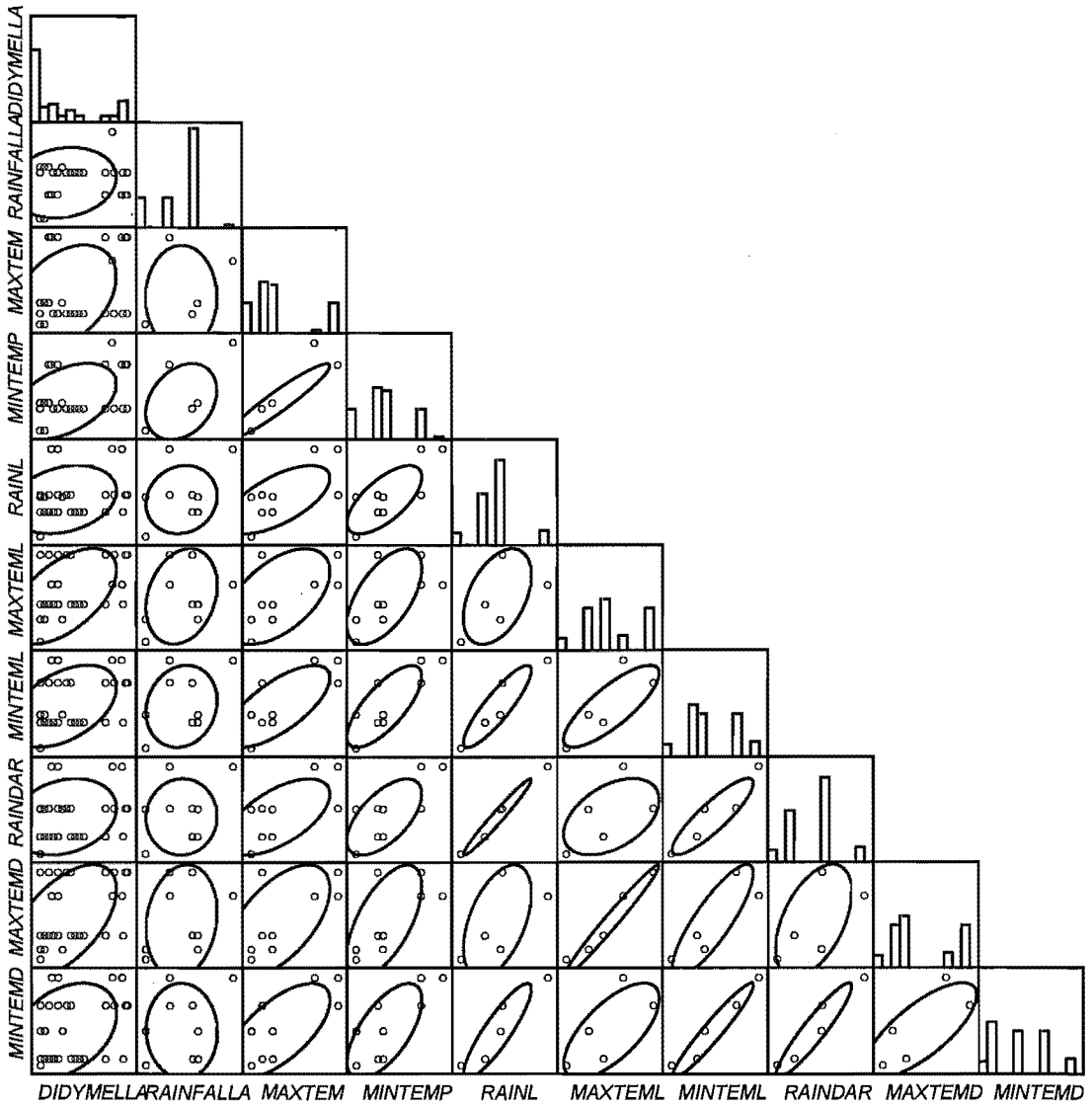

Figure 2.6 Spearman rank correlations of environmental factors measured at three NIWA weather station sites and *Didymella* levels. 95% Confidence interval indicated by ellipsoidal shape. Station codes are A, Ashburton Township, L, Lincoln; D, Darfield.

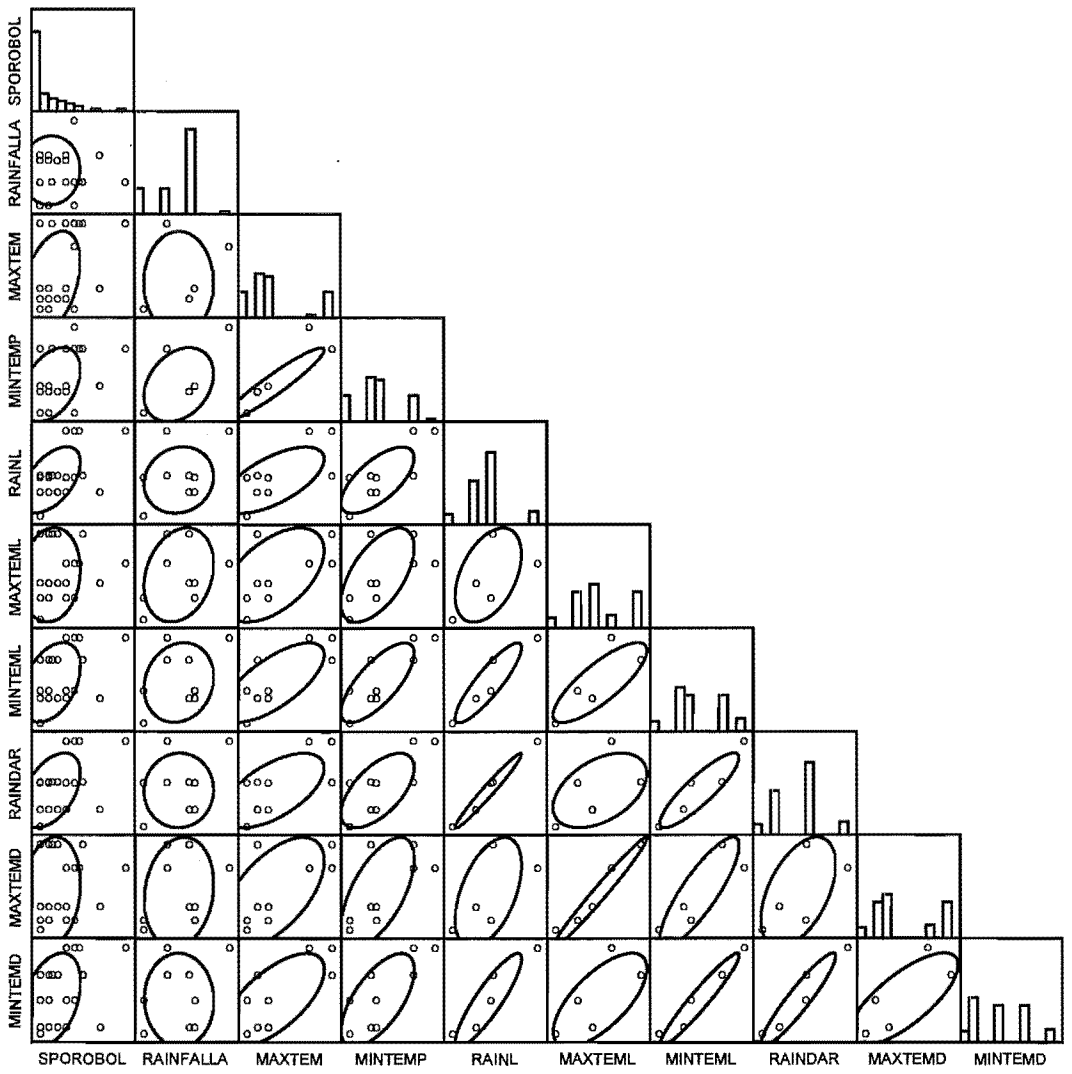

Figure 2.7 Spearman rank correlations of environmental factors measured at three NIWA weather station sites and *Sporobolomyces* spp. levels. 95% Confidence interval indicated by ellipsoidal shape. Station codes are A, Ashburton Township, L, Lincoln; D, Darfield.

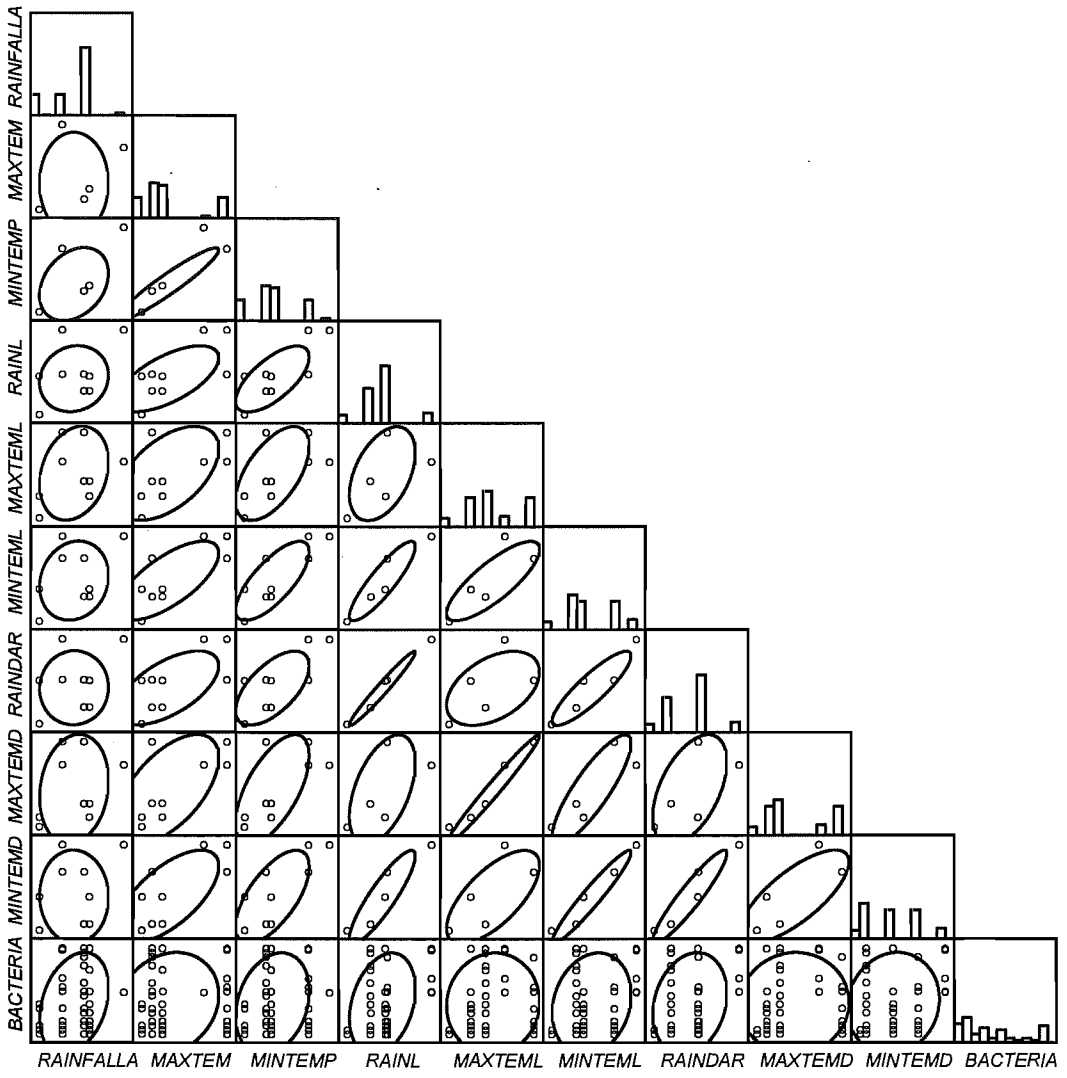

Figure 2.8 Spearman rank correlations of environmental factors measured at three NIWA weather station sites and Bacteria spp. levels. 95% Confidence interval indicated by ellipsoidal shape. Station codes are A, Ashburton Township, L, Lincoln; D, Darfield.

Table 2.1 Significance table (P<0.05) of the effects of fungicide treatment (tebuconazole or azoxystrobin) or the untreated control and trial (Methven 95/96 and 96/97, Chertsey 95/96 and Pendarvis 96/97) on the levels of *Didymella*, Bacteria and *Sporobolomyces*, isolated from surface sterilised green leaf tissue.

	Treatment	**Trials**	**Interaction between Treatment and trials**
Didymella	ns	ns	ns
Bacteria	ns	ns	ns
Sporobolomyces	ns	ns	ns

1995/96

Table 2.2 Isolation of micro-organisms from surface sterilised wheat leaves (leaf positions 1,5,8) at 11 sampling dates from untreated plots in a field trial at Methven in 1995/96. The sampling dates were 1, 21/9/95, 2, 1/10/95, 3, 11/10/95, 4, 22/10/95, 5, 22/11/95, 6, 20/11/95, 7, 26/11/95, 8, 30/11/95, 9, 4/12/95, 10, 11/12/95, 11, 18/12/95.

Taxa	**Percentage Infection of Leaves for Differing Leaf Stages (Percentage Leaf Pieces Infected in Brackets)**											
	Leaf 1						Leaf 5			Leaf 8		
Sampling dates	1	2	3	4	5	6	5	6	7	9	10	11
Didymella sp.	0	4 (4)	35 (5)	15 (3)	10 (3)	0	5 (5)	30 (9)	5 (2)	0	0	15 (8)
Alternaria sp.	0	30 (11)	60 (21)	10 (6)	25 (10)	65 (27)	25 (4)	70 (28)	20 (4)	0	50 (15)	100 (80)
Septoria nodorum	0	0	0	0	0	0	0	0	0	0	5 (1)	0
Fusarium sp.	0	0	0	0	10 (2)	0	10 (2)	0	5 (1)	0	0	0
Cladosporium sp.	0	0	0	0	0	0	0	5 (2)	0	0	5 (1)	0
Sporobolomyces sp.	0	0	0	0	5 (1)	0	0	20 (5)	10 (6)	0	0	5 (1)
Penicillium sp.	0	1 (3)	0	5 (1)	20 (6)	0	20 (4)	0	5 (3)	0	0	5 (1)
Helminthosporium sp.	0	1 (1)	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	0	0	0	0	0	5 (4)	0	0
Mycelia sterile	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria sp.	0	10 (1)	5 (5)	25 (11)	40 (13)	90 (55)	20 (7)	95 (71)	95 (83)	5 (4)	0	5 (2)

Table 2.3 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Methven trial during 1995/96. Treatments are: 1, water control; 2, tebuconazole applied at GS 59; 3, azoxystrobin applied at GS 59.

Taxa	Percentage Infection of Leaves		
	Treatment		
	1[1]	2[1]	3[1]
Didymella sp.	25	75	0
Alternaria sp.	100	100	100
Septoria nodorum	25	75	0
Fusarium sp.	0	0	0
Cladosporium sp.	50	0	0
Sporobolomyces sp.	25	0	0
Penicillium sp.	25	75	25
Helminthosporium sp.	0	0	0
Phoma sp.	0	0	0
Mycelia sterile	0	0	0
Bacteria sp.	25	75	25

[1] Refers to treatment type

Table 2.4 Isolation of micro-organisms from surface sterilised wheat leaves (leaf positions 1,5,8) at 10 sampling dates from untreated plots in a field trial at Mitcham in 1995/96. The sampling dates were 1, 21/9/95, 2, 1/10/95, 3, 11/10/95, 4, 22/10/95, 5, 22/11/95, 6, 20/11/95, 7, 26/11/95, 8, 30/11/95, 9, 4/12/95, 10, 11/12/95.

Leaf Stages / Taxa	Percentage Infection of Leaves for Differing Leaf Stages (Percentage Leaf Pieces Infected in Brackets)													
	Leaf 1						Leaf 5			Leaf 8				
	1	2	3	4	5	6	5	6	7	6	7	8	9	10
Didymella sp.	0	20 (6)	20 (11)	55 (24)	45 (12)	30 (13)	5 (1)	20 (2)	20 (5)	5 (2)	0	0	0	0
Alternaria sp.	0	25 (6)	50 (23)	35 (7)	80 (43)	65 (38)	5 (1)	25 (7)	35 (19)	0	30 (6)	15 (3)	0	10 (3)
Septoria nodorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	5 (1)	5 (1)	0	0	0	0	0	0	0	0
Cladosporium sp.	0	15 (3)	10 (2)	0	15 (4)	5 (1)	0	0	5 (1)	0	0	5 (1)	0	0
Sporobolomyces sp.	0	0	5 (2)	5 (1)	0	0	0	0	15 (5)	0	0	0	0	10
Penicillium sp.	0	0	10 (3)	20 (6)	0	15 (4)	0	15 (3)	15 (3)	5 (1)	10 (2)	5 (4)	0 (1)	40 (9)
Helminthosporium sp.	0	5	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	5 (1)	0	0	0	0	0	0	0	0	0
Mycelia sterile	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria sp.	0	10 (15)	20 (10)	35 (7)	20 (6)	40 (9)	0	35 (17)	95 (21)	5 (2)	40 (11)	30 (7)	0	55 (7)

Table 2.5 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Mitcham trial during 1995/96. Treatments are: 1, water control; 2, tebuconazole at GS 59; 3, azoxystrobin at GS 59.

Taxa	**Percentage Infection of Flag Leaves**		
	Treatment		
	1	2	3
Didymella sp.	10	0	0
Alternaria sp.	100	10	30
Septoria nodorum	5	0	0
Fusarium sp.	0	0	0
Cladosporium sp.	5	0	0
Sporobolomyces sp.	0	0	0
Penicillium sp.	5	5	5
Helminthosporium sp.	0	0	0
Phoma sp.	5	0	0
Mycelia sterile	0	0	0
Bacteria sp.	10	20	10

Table 2.6 Isolation of micro-organisms from surface sterilised wheat leaves (leaf positions 1,5,8) at 11 sampling dates from untreated plots in a field trial at Chertsey in 1995/96. The sampling dates were 1, 21/9/95, 2, 1/10/95, 3, 11/10/95, 4, 22/10/95, 5, 22/11/95, 6, 20/11/95, 7, 26/11/95, 8, 30/11/95, 9, 4/12/95, 10, 11/12/95, 11, 18/12/95.

Taxa	**Percentage Infection of Leaves for Differing Leaf Stages (Percentage Leaf Pieces Infected in Brackets)**														
	Leaf 1						Leaf 5				Leaf 8				
Sampling times	1	2	3	4	5	6	6	7	8	9	7	8	9	10	11
Didymella sp.	0	5 (1)	5 (14)	5 (1)	5 (2)	20 (10)	10 (4)	0	0	15 (4)	20 (5)	5 (2)	0	0	15 (15)
Alternaria sp.	0	65 (22)	15 (24)	10 (2)	15 (4)	65 (32)	55 (27)	0	5 (1)	10 (4)	20 (4)	30 (6)	70 (57)	90 (85)	100 100
Septoria nodorum	0	0	0	0	0	0	0	0	0	0	0	0	5 (1)	0	0
Fusarium sp.	0	15 (3)	5 (1)	0	0	0	0	0	0	5 (1)	0	5 (1)	0	0	0
Cladosporium sp.	0	5 (1)	0	0	0	15 (5)	0	0	0	0	0	0	0	5 (5)	20 (20)
Sporobolomyces sp.	0	0	0	5 (2)	0	5 (1)	0	0	0	0	0	0	0	0	0
Penicillium sp.	0	10 (2)	5 (1)	40 (12)	0	10 (2)	20 (5)	0	0	15 (4)	0	10 (2)	0	10 (10)	5 (5)
Helminthosporium sp.	0	5 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mycelia sterile	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria sp.	0	30 (6)	15 (5)	55 (21)	65 (40)	75 (21)	85 (20)	55 (11)	0	70 (31)	70 (18)	75 (42)	75 (37)	0	5 (5)

Table 2.8 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Chertsey trial during 1995/96. Treatments are: 1, water control; 2, tebuconazole at GS 47; 3, azoxystrobin at GS 47, 4, water control, 5, tebuconazole at GS 59, 6, azoxystrobin at GS 59, 7, water control, 8, tebuconazole at GS 65, 9, azoxystrobin at GS 65.

Taxa	Percentage Infection of Leaves								
	Treatment								
	1	2	3	4	5	6	7	8	9
Didymella sp.	100	25	0	25	75	0	0	0	0
Alternaria sp.	100	100	75	100	100	100	100	100	100
Septoria nodorum	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	25	0	0	0	0	0	0
Cladosporium sp.	50	75	25	75	75	50	100	50	25
Sporobolomyces sp.	0	0	0	0	0	0	0	0	0
Penicillium sp.	100	75	100	100	100	100	100	100	100
Helminthosporium sp.	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	0	0	0	0	0
Mycelia sterile	0	0	0	0	0	0	0	0	0
Bacteria sp.	100	75	100	75	50	100	50	75	100

Table 2.7 Isolation of micro-organisms from surface sterilised wheat leaves (leaf positions 1,5,8) at 13 sampling dates from untreated plots in a field trial at Norwood in 1995/96. The sampling dates were 1, 21/9/95, 2, 1/10/95, 3, 11/10/95, 4, 22/10/95, 5, 22/11/95, 6, 20/11/95, 7, 26/11/95, 8, 30/11/95, 9, 4/12/95, 10, 11/12/95, 11, 18/12/95, 12, 2/1/96, 13 8/1/96.

Taxa	**Percentage Infection of Leaves for Differing Leaf Stages (Percentage Leaf pieces Infected in Brackets)**															
	Leaf 1					Leaf 5					Leaf 8					
Sampling times	1	2	3	4	5	6	7	8	9	10	7	8	9	10	11	12	13
Didymella sp.	0	15 (5)	20 (5)	25 (5)	10 (5)	0	0	10 (2)	25 (16)	5 (5)	0	0	0	0	0	35 (25)	25 (8)
Alternaria sp.	0	25 (8)	65 (18)	30 (10)	20 (6)	0	5 (1)	0	10 (5)	50 (24)	5 (1)	0	5 (1)	90 (67)	15 (5)	95 (62)	94 (62)
Septoria nodorum	0	0	0	0	5 (1)	0	0	0	0	5 (1)	0	0	0	0	5 (1)	0	0
Fusarium sp.	0	15 (4)	0	0	0	0	0	0	0	0	0	0	0	0	0	5 (5)	0
Cladosporium sp.	0	0	5 (1)	0	0	0	0	0	0	0	0	0	0	5 (5)	5 (3)	15 (3)	90 (36)
Sporobolomyces sp.	0	0	10 (4)	5 (1)	0	0	0	0	5 (5)	0	0	0	0	0	0	0	0
Penicillium sp.	0	5 (15)	0	0	5 (1)	5 (1)	0	15 (3)	10 (2)	10 (2)	0	5 (1)	5 (5)	0	10 (9)	0	0
Helminthosporium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	0	0	0	0	5 (1)	5 (1)	0	0	0	0	5 (1)	25 (15)	0
Mycelia sterile	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria sp.	0	45 (4)	30 (10)	75 (19)	70 (68)	0	5 (1)	55 (37)	95 (67)	10 (2)	5 (1)	15 (3)	10 (2)	10 (7)	100 (69)	0	15 (7)

Table 2.9 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Norwood trial during 1995/96. Treatments are: 1, water control; 2, tebuconazole at GS 47; 3, azoxystrobin at GS 47, 4, water control, 5, tebuconazole at GS 59, 6, azoxystrobin at GS 59, 7, water control, 8, tebuconazole at GS 65, 9, azoxystrobin at GS 65.

Taxa	**Percentage Infection of Flag Leaves**								
	Treatment								
	1	2	3	4	5	6	7	8	9
Didymella sp.	75	75	0	25	25	0	50	100	0
Alternaria sp.	100	75	100	100	100	100	100	100	100
Septoria nodorum	0	0	0	0	0	0	25	0	0
Fusarium sp.	75	25	0	25	75	0	25	0	25
Cladosporium sp.	50	0	0	0	25	50	25	50	0
Sporobolomyces sp.	25	0	0	0	0	0	25	0	0
Penicillium sp.	100	30	75	75	0	25	50	25	0
Helminthosporium sp.	0	0	0	0	0	0	0	0	0
Phoma sp.	0	50	25	25	25	25	25	50	25
Mycelia sterile	0	0	0	0	0	0	0	0	0
Bacteria sp.	75	100	100	25	100	100	50	25	0

Table 2.10 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Aylesbury trial during 1996/97. Treatments are: 1, water control; 2, tebuconazole at GS 49; 3, azoxystrobin at GS 49, 4, water control, 5, tebuconazole at GS 65, 6, azoxystrobin at GS 65. Sample number: 1, collected 19/11/96, 2, collected 2/12/96, 3, collected 16/12/96 9, azoxystrobin at GS 65.

Taxa	**Percentage Infection of Flag Leaves**											
	Treatment 1			Treatment 2			Treatment 3			Treatment 4	Treatment 5	Treatment 6
Sampling times	1	2	3	1	2	3	1	2	3	3	3	3
Didymella sp.	0	0	60	0	4	50	0	0	22	100	90	0
Alternaria sp.	20	5	40	0	10	30	0	0	44	21	30	10
Septoria nodorum	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium sp.	0	0	20	0	0	0	0	0	0	0	0	0
Sporobolomyces sp.	0	0	20	0	4	0	0	0	0	14	10	20
Penicillium sp.	0	0	75	0	0	20	0	0	0	0	10	0
Helminthosporium sp.	0	0	0	0	0	0	0	0	11	0	0	0
Phoma sp.	0	0	20	0	0	0	0	0	0	7	10	0
Mycelia sterile	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria sp.	6	5	0	0	0	30	10	0	22	0	40	60

Table 2.11 Isolation of micro-organisms from surface sterilised wheat leaves (leaf positions 1-8) at eight sampling dates from untreated plots in a field trial at Pendarvis 1996/97. Sampling times are: 1, 23/9/96, 2, 7/10/96, 3, 22/10/96, 4, 5/11/96, 5, 19/11/96, 6, 2/12/96, 7, 16/12/96, 8, 2/1/97.

Taxa	Percentage Infection of Leaves for Differing Leaf Stages (Percentage Leaf pieces Infected in Brackets)																						
	Leaf 1			Leaf 2			Leaf 3				Leaf 4			Leaf 5			Leaf 6			Leaf 7				Leaf 8
Sampling times	1	2	3	2	3	4	2	3	4	5	3	4	5	4	5	6	4	5	6	5	6	7	8	6
Didymella sp.	0	0	25 (5)	0	0	5 (1)	0	0	0	50 (11)	0	0	40 (18)	10 (3)	95 (34)	100 (68)	0	75 (32)	100 (59)	35 (13)	85 (30)	100 (52)	20 (4)	30 (7)
Alternaria sp.	0	30 (6)	50 (14)	5 (1)	10 (2)	30 (8)	0	15 (3)	15 (3)	15 (3)	10 (2)	25 (7)	30 (6)	10 (2)	30 (6)	0	0	30 (9)	20 (9)	25 (5)	15 (6)	20 (4)	0	0
Septoria nodorum	0	0	5 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	0	0	0	5 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5 (1)	0
Cladosporium sp.	0	0	0	0	5 (1)	15 (3)	0	5 (1)	0	5 (1)	0	0	5 (1)	0	15 (5)	15 (3)	0	5 (1)	5 (1)	0	0	0	0	10 (2)
Sporobolomyces sp.	0	0	15 (6)	0	20 (4)	5 (1)	0	0	35 (10)	10 (2)	0	0	5 (1)	0	5 (1)	0	0	10 (2)	10 (2)	5 (1)	10 (2)	0	20 (8)	0
Penicillium sp.	0	10 (2)	10 (3)	0	10 (2)	5 (1)	0	10 (2)	5 (2)	15 (3)	10 (2)	15 (3)	10 (2)	30 (6)	10 (2)	0	0	10 (2)	0	5 (1)	0	15 (3)	0	0
Helminthosporium sp.	0	0	5 (1)	0	5 (1)	0	0	0	0	0	0	5 (1)	0	0	0	0	0	0	5 (1)	0	0	0	0	0
Phoma sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15 (4)	0	0	0	0	0	10 (2)	5 (1)	0
Mycelia sterile	0	0	0	0	5 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5 (1)
Bacteria sp.	0	10 (3)	50 (12)	5 (1)	35 (7)	100 (89)	0	25 (5)	100 (97)	100 (71)	10 (3)	75 (35)	80 (44)	25 (6)	45 (27)	30 (9)	0	45 (14)	90 (51)	25 (10)	15 (3)	30 (14)	100 (96)	30 (9)

Table 2.12 Isolation of micro-organisms from surface sterilised wheat leaves (leaf positions 1-8) at eight sampling dates from untreated plots in a field trial at Methven 1996/97. Sampling times are: 1, 23/9/96, 2, 7/10/96, 3, 22/10/96, 4, 5/11/96, 5, 19/11/96, 6, 2/12/96, 7, 16/12/96, 8, 2/1/97.

Taxa	Percentage Infection of Leaves for Differing Leaf Stages (Percentage Leaf pieces Infected in Brackets)																						
	Leaf 1			Leaf 2			Leaf 3				Leaf 4			Leaf 5				Leaf 6			Leaf 7			Leaf 8
Sampling times	1	2	3	2	3	4	2	3	4	5	3	4	5	4	5	6	7	5	6	7	6	7	8	6
Didymella sp.	0	5 (1)	0	0	0	0	0	0	0	40 (9)	0	5 (1)	45 (10)	0	20 (4)	35 (13)	10 (4)	15 (3)	0	100 (51)	0	75 (41)	94 (42)	0
Alternaria sp.	0	15 (7)	15 (5)	0	25 (6)	10 (3)	0	0	5 (1)	0	15 (3)	0	40 (10)	0	25 (7)	15 (3)	0	10 (3)	0	5 (1)	0	15 (3)	53 (17)	0
Septoria nodorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5 (1)	0	0	0	0	0	0	12 (2)	0
Cladosporium sp.	0	0	5 (1)	0	0	0	0	0	0	5 (1)	0	0	0	0	10 (2)	0	0	0	0	0	0	0	63 (25)	0
Sporobolomyces sp.	0	5 (1)	0	0	0	0	0	15 (3)	0	15 (3)	0	0	5 (1)	0	0	5 (1)	0	0	0	25 (5)	0	0	50 (11)	0
Penicillium sp.	0	5 (1)	0	5 (1)	25 (6)	5 (1)	15 (3)	10 (2)	0	0	0	50 (11)	0	5 (1)	15 (3)	5 (1)	0	5 (1)	0	0	5 (1)	0	35 (7)	0
Helminthosporium sp.	0	0	0	0	0	0	0	0	0	5 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	10 (3)	0	0	5 (1)	0	0	0	0	0	0	0	0	0	15 (3)	0	0	0	5 (1)	0	0	0	0
Mycelia sterile	0	0	5 (1)	0	5 (1)	20 (4)	0	0	0	0	0	0	0	0	0	0	0	20 (4)	0	5 (1)	0	0	0	0
Bacteria sp.	0	30 (8)	100 (81)	10 (2)	15 (6)	100 (84)	5 (1)	10 (2)	35 (10)	95 (41)	15 (3)	5 (1)	60 (19)	0	10 (3)	50 (17)	10 (5)	10 (2)	0	5 (1)	10 (2)	15 (8)	65 (25)	0

Table 2.13 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Methven trial during 1996/97. Treatments are: 1, water control; 2, tebuconazole at GS 49; 3, azoxystrobin at GS 49, 4, water control, 5, tebuconazole at GS 65, 6, azoxystrobin at GS 65. Sample number: 1, collected 16/12/96, 2, collected 2/1/97, 3, collected 22/1/97.

Taxa	**Percentage Infection of Flag Leaves**														
	Treatment 1			Treatment 2			Treatment 3			Treatment 4		Treatment 5		Treatment 6	
Sample number	1	2	3	1	2	3	1	2	3	1	2	2	3	2	3
Didymella sp.	20	35	65	20	35	100	10	15	30	5	100	20	93	0	5
Alternaria sp.	0	10	10	15	10	30	0	20	65	0	33	15	20	20	45
Septoria nodorum	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	10	0	0	10	0	0	20	0	6	0	0	0	15
Cladosporium sp.	0	20	100	0	10	80	0	10	95	0	77	10	87	0	70
Sporobolomyces sp.	0	45	40	0	35	0	0	40	5	0	0	50	6	10	5
Penicillium sp.	15	35	25	10	0	5	5	30	40	5	5	0	6	0	30
Helminthosporium sp.	0	10	0	0	5	0	0	5	0	0	0	0	0	0	0
Phoma sp.	5	10	5	0	0	5	0	5	5	0	6	0	0	5	0
Mycelia sterile	0	0	5	0	0	10	5	5	0	0	12	0	18	0	5
Bacteria sp.	0	95	10	5	100	30	5	90	90	100	87	100	80	100	100

Table 2.14 The effects of the fungicides tebuconazole and azoxystrobin on the levels of various fungal taxa and bacterial numbers isolated from surface sterilised flag leaves of wheat at the Pendarvis trial during 1996/97. Treatments are: 1, water control; 2, tebuconazole at GS 49; 3, azoxystrobin at GS 49, 4, water control, 5, tebuconazole at GS 65, 6, azoxystrobin at GS 65. Sample number: 1, collected 2/12/96, 2, collected 16/12/96, 3, collected 2/1/97.

Taxa	**Percentage infection of Flag Leaves**														
	Treatment 1			Treatment 2			Treatment 3			Treatment 4		Treatment 5		Treatment 6	
Sample number	1	2	3	1	2	3	1	2	3	2	3	2	3	2	3
Didymella sp.	50	95	20	0	85	35	0	80	40	100	5	90	35	95	0
Alternaria sp.	0	15	10	0	10	55	0	0	55	7	25	0	45	25	15
Septoria nodorum	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0
Fusarium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium sp.	0	0	55	0	0	40	0	0	70	7	0	0	60	0	30
Sporobolomyces sp.	0	0	10	0	10	20	0	0	45	14	20	0	25	0	10
Penicillium sp.	0	15	25	0	0	20	0	5	40	7	0	20	55	20	15
Helminthosporium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	0	0	5	5	0	5	0	0	0	6	10	0	0
Mycelia sterile	0	0	5	0	0	0	0	0	0	0	0	6	0	5	5
Bacteria sp.	0	45	70	0	40	85	0	55	50	64	100	38	55	0	100

2.203 Field survey

2.2031 1995/96

Table 2.15 Percentage of wheat and barley crops surveyed during the 1995/96 field survey that were affected by *Didymella*, and percentage of leaf area affected.

Host Species	Number of crops surveyed	Percentage of crops affected by *Didymella*	Percentage leaf area affected
wheat	14	70	2.8
barley	4	20	1

As can be seen from Table 2.15, 70% of surveyed wheat crops had *Didymella* present affecting, on average 2.8% of the leaf area. Only 20% of barley crops surveyed were affected by *Didymella,* and the level of fruiting bodies on the leaf was 1%.

2.2032 1996/97

Eleven percent of the leaf area was affected in 97% of wheat crops surveyed (Table 2.17) in the 1996/97 survey. Seventy-three percent of barley crops surveyed were infected and the leaf area affected was on average 4.6%. As can be seen from Table 2.17, oats and triticale crops were also affected. Other pathogens, for example, *S. nodorum* and *S. tritici*, were present in under 20% of crops and affected two percent of less of the leaf area (Table 2.16). *Didymella* was relatively common on grasses present at the crop margins and often on wild oat plants present within crops (Table 2.16).

Table 2.16 The average percentage of crops and grasses, and the percentage of leaf area affected by *Didymella* spp., *S. nodorum*, and *S. tritici* in the 1996/97 field survey.

Pathogen	Average percentage of crops and grasses affected	Average percentage leaf area affected
Didymella spp.	84	9.1
Septoria nodorum	18	2.0
Septoria tritici	14.3	0.83

Table 2.17 The percentage of wheat, barley, oats and triticale crops found to be affected by *Didymella* and the mean percentage leaf area affected during the 1996/97 field survey.

Host Spp.	Number of crops surveyed	Percentage of crops affected by *Didymella*	Percentage leaf area affected
wheat	38	97	11.4
barley	15	73	4.6
oats	2	100	4.6
triticale	1	100	3.7

2.2033 Grasses present at the crop margins

Grasses present at the crop margins of the four trials during the 1995/96 growing season had pseudothecia of *Didymella* affecting up to 70% of the leaves examined (Table 2.18). There were five species of grass present. The only other pathogen identified was *S. tritici* which was present on 1% of leaves of *H. murinum*, *D. glomerata* and *B. mollis* examined present trials

Table 2.18 The average percentage of leaves and leaf area of various grasses affected by *Didymella* spp. and *S. tritici* present at the crop margins of four trials (Chertsey, Mitcham, Methven and Norwood) at three sampling times during 1995/96.

Date	Site	Grass spp.	Percentage leaves affected by *Didymella* spp. (average % leaf area affected)	Percentage leaves affected by *S. tritici* (average % leaf area affected)
20/11/95	Mitcham	*Hordeum murinum*	30 (1.6)	0
		Bromus mollis	0	0
4/12/95	Methven	*Hordeum murinum*	70 (9.55)	0
		Bromus mollis	30 (0.7)	1 (0.15)
11/12/95	Methven	*Hordeum murinum*	40 (0.68)	1 (0.15)
		Dactylis glomerata	50 (0.5)	1 (0.05)
		Bromus diandrus	40 (0.8)	0
11/12/95	Mitcham	*Festuca arundinacea*	10 (0.1)	0
11/12/95	Norwood	*Festuca arundinacea*	30 (0.7)	0
11/12/95	Chertsey	*Festuca arundinacea*	40 (0.8)	0

2.2034 Resistance

2.20341 1995/96 and 1996/97

Table 2.19 Table showing statistically significant differences between the effects of both trial (Chertsey 1995/96, Methven 1995/96, Methven 1996/97, Pendarvis 1996/97) and year (1995/96 and 1996/97). ($p<0.001$) for the percentage leaf area affected by *Didymella* spp.

Source	Significance
cultivars	ns
trials	***
year	***

The levels of *Didymella* sporulation on senesced leaf tissue differed significantly between trials (Table 2.19). Levels of *Didymella* were significantly higher on cultivars during the 1996/97 growing season than during 1995/96. There were no differences in the level of *Didymella* sporulation on any of the cultivars examined during both growing seasons (Figure 2.9 and 2.10). The level of sporulation was highest on cultivars grown at the Methven trial site during 1996/97.

2.2035 Sporulation of *Didymella* on senesced leaf material

2.20351 1995/96

Table 2.20 The differences between trials sited at Chertsey, Methven, Mitcham and Norwood in the percentage of senesced wheat (cv. Monad) leaf area affected by *Didymella* during the 1995/96 growing season

Trials	
Chertsey	a
Methven	b
Mitcham	b
Norwood	a

Letters indicate differences as determined by Tukey's multiple range test ($p<0.05$)

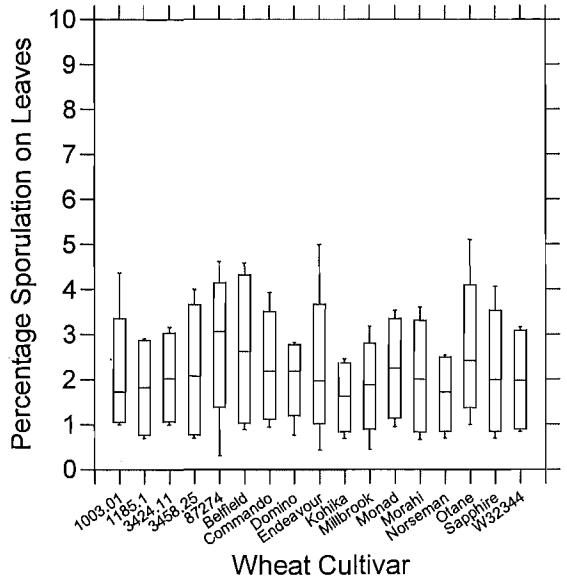

Figure 2.9 Percentage sporulation of *Didymella* spp. on differing wheat cultivars for the 1995/96 growing season. Box and whisker plots show median value, inter-quartile range, and minimum and maximum values.

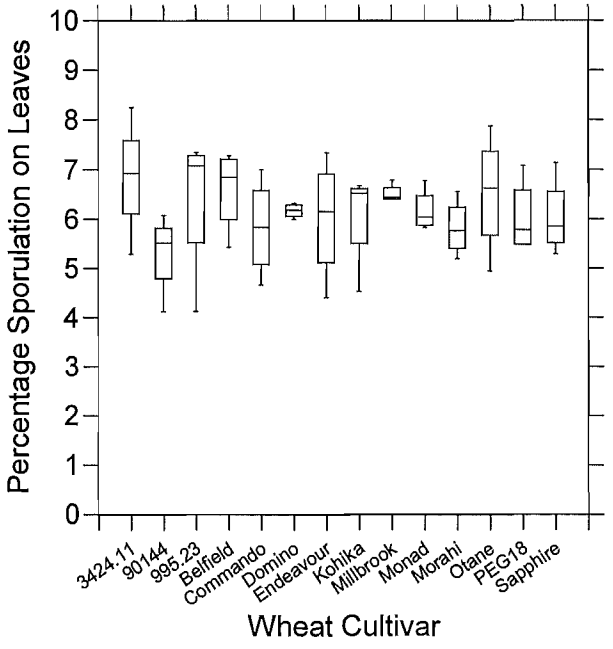

Figure 2.10 Percentage sporulation of *Didymella* spp. on differing wheat cultivars for the 1996/97 growing season. Box and whisker plots show median value, inter-quartile range, and minimum and maximum values.

Table 2.21 The level of *Didymella* sporulation on senesced leaf material (1995/96) that had been treated with either a water application, or the application of either tebuconazole or azoxystrobin. Treatments: 1, 4, and 7 are untreated controls, 2, 5, and 8 are tebuconazole applied at GS 47, 59 and 65 respectively, and 3, 6 and 9 are azoxystrobin applied at GS 47, 59 and 65 respectively. Data is pooled over the four trials.

Treatment	1	2	3	4	5	6	7	8
1								
2	ns							
3	***	***						
4	ns	***	ns					
5	ns	ns	***	ns				
6	***	***	ns	ns	***			
7	ns	***	ns	ns	ns	ns		
8	***	***	ns	ns	***	ns	ns	
9	***	***	ns	ns	***	ns	ns	ns

ns: not significant ($p \leq 0.05$)
***: significant $p \leq 0.001$

The Chertsey and Norwood trials had significantly lower levels of *Didymella* sporulation than the two situated at Mitcham and Methven (Table 2.20). The effect of treatment is significant. The highest levels of sporulation were recorded in the control and tebuconazole (GS 47) treatment, whilst the lowest levels were recorded in treatments three (azoxystrobin GS 47), six (azoxystrobin GS 59), eight (tebuconazole GS 65) and nine (azoxystrobin GS 65) (Table 2.21).

2.20352 1996/97

Table 2.22 The differences between trials sited at Aylesbury, Highbank, Methven and Pendarvis, in the percentage of senesced wheat (cv. Monad) leaf area affected by *Didymella* during the 1996/97 growing season

Source	Significance
Aylesbury	a
Highbank	b
Methven	b
Pendarvis	C

Table 2.23 The level of *Didymella* sporulation on senesced leaf material (1996/97) that had been treated with either a water application, or the application of either tebuconazole or azoxystrobin. Treatments: 1, and 4 are untreated controls, 2 and 5 are tebuconazole applied at GS 49 and 65 respectively, and 3 and 6 are azoxystrobin applied at GS 49 and 65 respectively. Data is pooled over the four trials.

Treatment	1	2	3	4	5
1					
2	ns				
3	***	***			
4	ns	ns	***		
5	ns	ns	***	ns	
6	***	***	ns	***	***

ns: not significant ($p \leq 0.05$)

***: significant $p \leq 0.001$

The level of *Didymella* sporulation was lowest at Aylesbury and highest at Pendarvis (Table 2.22). The sporulation of *Didymella* on leaves treated with tebuconazole was significantly higher than that on those treated with azoxystrobin at both fungicide application timings or vice versa (Table 2.23). At Aylesbury, Pendarvis, and Methven the azoxystrobin treated leaves had significantly less *Didymella* sporulating than the untreated control leaves. There was also a significant interaction effect between the amount of *Didymella* sporulating on the leaf tissue and the trial as higher levels were present in the Methven trial than the Aylesbury trial.

2.2036 Presence of *Sporobolomyces* in 1996/97 and 1997/98

The levels of *Sporobolomyces* on fungicide treated leaves was not significantly different from the untreated control leaves. Leaf surface orientation also did not affect the numbers of *Sporobolomyces* colonies isolated (Table 2.24).

Table 2.24 The effect of treatment, replicate and leaf surface orientation and the interaction between these variables on the number of *Sporobolomyces* colonies isolated from three trials sited at Pendarvis (1996/97), Methven (1996/97) and Hilton (1997/98) ($p \leq 0.05$).

Trial	Treatment	Leaf orientation	Replicate	Interaction between Treatment and leaf orientation	Interaction between Treatment and replicate	Interaction between Treatment, replicate and leaf orientation
Pendarvis	ns	ns	ns	ns	ns	Ns
Methven	ns	ns	ns	ns	ns	Ns
Hilton	ns	ns	ns	ns	ns	Ns

2.2037 Seed plating

Penicillin was the only fungal genus isolated from the surface sterilised wheat grains. Grains that were artificially inoculated with *Didymella* germinated at the same rate as uninoculated grains, and grew into apparently healthy plants. The seeds that were plated with the four *Didymella* isolates had healthy hypocotyls and did not appear diseased nor was there any evidence of sporulation. *Didymella* could be isolated from the leaf material of these plants, and the rate of senescence was not statistically significant from the uninoculated controls.

2.2038 Antagonism

When *Didymella* was plated together with other commonly isolated fungi and bacteria from leaf laminar it was typically overgrown (type 2b) by the species under investigation. *Fusarium* spp., *Alternaria* spp., *Helminthosporium* spp., *S. nodorum* and *Phoma* spp. all over-grew the *Didymella* isolates which stopped growing once the colony diameter reached 10 mm. Bacterial isolates reduced the growth of *Didymella* but were not significantly different from the control plates which only had *Didymella* plated out. *Sporobolomyces* had no effect on *Didymella*.

2.2039 High humidity and leaf imprinting

The only fungal genera that was identified using high humidity was *Alternaria*. It tended to overgrow the leaf material thus masking the development of any other fungi.

The levels of the species isolated fluctuated except bacterial levels which increased (Tables 2.25, 2.26). Bacterial levels were not able to be enumerated due to the spreading nature of the colonies. There were no observable increases in any species as the leaves aged. *Didymella* was isolated only once. The leaf imprinting method had to be stopped after the sixth sample as bacterial levels had increased making the identification of any fungi almost impossible. *Penicillium* spp. was the only fungus consistently isolated.

Table 2.25 The fungal species isolated from the phylloplane of leaves 1-8 of wheat cv. Monad using a leaf imprinting technique over four sampling times ((3) 7/10/96, (4) 5/11/96, (5) 19/11/96, (6) 2/12/96)at the Pendarvis trial during 1996/97

Date	Leaf Position	*Penicillium* spp.	*Alternaria* spp.	*Cladosporium* spp.	*Didymella* spp.	*Fusarium* spp.	*Phoma* spp.	*Sporobolomyces*
3	1	10	2	3	1	1	4	0
4	1	11	0	3	0	0	1	0
	2	9	3	0	0	0	0	4
	3	12	0	2	0	0	1	8
	4	15	2	2	0	0	0	6
5	2	1	1	2	0	0	0	4
	3	0	0	0	0	0	1	0
	4	0	0	0	0	0	0	0
6	5	8	2	0	0	0	1	2
	6	3	0	0	0	1	1	3
	7	7	2	0	0	0	0	1
	8	15	4	0	2	0	0	1

Table 2.26 The fungal species isolated from the phylloplane of leaves 1-8 of wheat cv. Monad using a leaf imprinting technique over four sampling times ((3) 7/10/96, (4) 5/11/96, (5) 19/11/96, (6) 2/12/96) at the Methven trial during 1996/97

Date	Leaf Position	*Penicillium* spp.	*Alternaria* spp.	*Cladosporium* spp.	*Didymella* spp.	*Fusarium* spp.	*Phoma* spp.	*Sporobolomyces*
3	1	12	3	1	0	0	0	2
4	1	7	7	0	0	0	0	
	2	22	2	2	0	0	0	14
5	3	0	1	0	0	0	1	5
	4	4	1	0	0	0	2	4
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	1	3
	7	0	0	0	0	0	0	0
6	5	8	0	0	0	0	5	46
	6	25	0	0	0	0	13	30
	7	28	2	0	1	0	8	7

The predominant bacterial colonies isolated from the phyllosphere were Gram negative rods.

2.2040 Embedded leaves

Few fungi apart from *Penicillium* spp. could be isolated from the embedded leaves on either Martins or nutrient agar. Whether this was due to the rapidity of the growth of the *Penicillium* spp. remains unknown. Pink and white yeast and Gram negative bacteria were isolated from every leaf that was embedded.

2.2041 Leaf clearing

This technique also proved relatively unsuccessful for the observing the presence of *Didymella* spores and other potential pathogens in the phylloplane prior to infection. Alternatively few pathogens were present. *Alternaria* spores were found on 95% of leaves examined as were yeasts (98% of leaves examined). One *Erysiphe graminis* var. *tritici* spore was found on the third leaf from the Pendarvis trial. *Cladosporium* was less common at 58% and *Epicoccum nigrum* at 21%. *Septoria tritici* was found on leaf two from the Methven trial at sample five and on the fifth leaf from the Pendarvis trial. *S. nodorum* spores were identified on the second leaf from the Pendarvis trial from the second sample. Also, from the second sample at the Pendarvis trial, a *Helminthosporium* spore was identified on the first leaf.

2.205 Indirect method of enumeration of phylloplane micro-organisms

2.2051 Leaf washings (Tables 2.27-2.51)

Bacterial and fungal numbers increased as the leaves aged. The only fungi commonly present were *Alternaria* and *Penicillium*. The levels of bacteria and fungi were reasonably similar on the media used during the 1995/96 season but not during the 1996/97 season. Bacterial levels were relatively low on newly expanded leaves but the levels increased as the leaves aged. Few yeast colonies were observed from the leaf washings. Martin's medium slowed the growth of *Alternaria*, *Penicillium* and bacteria allowing other slow growing fungi to grow. No bacteria were isolated from leaf seven from sample seven and eight from the Pendarvis trial. The application of fungicide sprays did not alter the number of bacteria and fungi present on leaf material. Fungicide application reduced levels of bacteria and fungi and levels remained lower than the controls during the period of sampling.

2.20511 1995/96

Chertsey

Table 2.27 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position one of wheat cv. Endeavour on PDA and Nutrient agar over eight sampling dates at the Chertsey trial during 1995/96. The sampling dates were 1, 21/9/95, 2, 1/10/95, 3, 11/10/95, 4, 12/10/95, 5, 22/11/95, 6, 20/11/95, 7, 26/11/95, 8, 30/11/95.

Date	Bacterial colonies/g fwt leaf tissue (PDA)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (PDA)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (PDA)	Yeast colonies/g fwt leaf tissue (NA)
1	20	10	0	0	0	0
2	0	40	0	40	280	0
3	1.1×10^3	165	0	0	0	0
4	1.2×10^4	3.7×10^2	300	0	10	0
5	4.2×10^4	4×10^4	0	0	0	0
6	6×10^3	1.2×10^5	4×10^3	0	0	0
7	6.9×10^5	5.6×10^5	0	0	0	0
8	1.8×10^5	1.7×10^5	0	0	0	0

Table 2.28 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position five of wheat cv. Endeavour on PDA and Nutrient agar over two sampling dates at the Chertsey trial during 1995/96. The sampling dates were 6, 20/11/95, 7, 26/11/95.

Date	Bacterial colonies/gf wt leaf tissue (PDA)	Bacterial colonies/gf wt leaf tissue (NA)	Fungal colonies/gf wt leaf tissue (PDA)	Fungal colonies/gf wt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (PDA)	Yeast colonies/g fwt leaf tissue (NA)
6	1.4×10^5	8×10^4	0	0	0	0
7	9.3×10^3	6.8×10^3	0	0	0	0

Table 2.29 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position eight of wheat cv. Endeavour on PDA and Nutrient agar over four sampling dates at the Chertsey trial during 1995/96. The sampling dates were 8, 30/11/95, 9, 4/12/95, 10, 11/12/95, 11, 18/12/95.

Date	Bacterial colonies/gf wt leaf tissue (PDA)	Bacterial colonies/gf wt leaf tissue (NA)	Fungal colonies/gf wt leaf tissue (PDA)	Fungal colonies/gf wt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (PDA)	Yeast colonies/g fwt leaf tissue (NA)
8	5.3×10^6	0	0	0	0	0
9	2.3×10^4	2.5×10^4	0	0	0	0
10	3.6×10^5	4.5×10^5	0	0	0	0
11	1.3×10^4	2.1×10^4	6.7×10^3	6.7×10^3	5.8×10^5	5.8×10^5

Methven

Table 2.30 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position one of wheat cv. Monad on PDA and Nutrient agar over eight sampling dates at the Methven trial during 1995/96. The sampling dates were 1, 21/9/95, 2, 1/10/95, 3, 11/10/95, 4, 22/10/95, 5, 12/11/95, 6, 20/11/95, 7, 26/11/95, 8, 30/11/95.

Date	Bacterial colonies/g fwt leaf tissue (PDA)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (PDA)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (PDA)	Yeast colonies/g fwt leaf tissue (NA)
1	0	10	0	10	0	0
2	328	40	240	10	20	200
3	147	0	70	0	20	0
4	1.6×10^3	500	2.8×10^3	0	0	0
5	7.4×10^3	3.7×10^3	0	1.3×10^3	0	0
6	2.7×10^3	6×10^3	0	0	0	0
7	6.8×10^4	5.6×10^4	0	0	0	0
8	7.4×10^3	$8x\ 10^4$	100	100	100	0

Table 2.31 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position five of wheat cv. Monad on PDA and Nutrient agar over two sampling dates at the Methven trial during 1995/96. The sampling dates were: 7, 26/11/95, 8, 30/11/95.

Date	Bacterial colonies/g fwt leaf tissue (PDA)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (PDA)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (PDA)	Yeast colonies/g fwt leaf tissue (NA)
7	8×10^4	5.1×10^4	0	0	0	0
8	$9.7\ 10^4$	2.6×10^4	100	0	0	0

Table 2.32 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position eight of wheat cv. Monad on PDA and Nutrient agar over three sampling dates at the Methven trial during 1995/96. The sampling dates were 9, 4/12/95, 10, 11/12/95, 11, 18/12/95.

Date	Bacterial colonies/g fwt leaf tissue (PDA)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (PDA)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (PDA)	Yeast colonies/g fwt leaf tissue (NA)
9	0	0	0	0	0	0
10	8.3×10^3	3.0×10^3	400	0	300	0
11	8.3×10^3	1.6×10^4	100	100	0	0

2.20512 1996/97

Pendarvis

Table 2.33 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position one of wheat cv. Monad on Martins medium and Nutrient agar over two sampling dates at the Pendarvis trial during 1996/97. Sampling dates were:, 3, 22/10/96, 4, 5/11/96,

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	1.3×10^4	0	0	0	0
4	0	987	1000	0	500	100

Table 2.34 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position two of wheat cv. Monad on Martins medium and Nutrient agar over two sampling dates at the Pendarvis trial during 1996/97. Sampling dates were: 3, 22/10/96, 4, 5/11/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	1.6×10^5	60	0	0	0
4	4×10^5	27	0	3.3×10^3	2.9×10^2	0

Table 2.35 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position three of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Pendarvis trial during 1996/97. Sampling dates were: 3, 22/10/96, 4, 5/11/96, 5, 19/11/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	1.3×10^3	0	0	150	0
4	3×10^3	73	0	0	0	0
5	2×10^4	2.1×10^4	0	0	95	0

Table 2.36 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position four of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Pendarvis trial during 1996/97. Sampling dates were: 3, 22/10/96, 4, 5/11/96, 5, 19/11/96,

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	1.8×10^5	0	0	0	0
4	1.8×10^3	4.1×10^3	0	0	75	38
5	256	2.5×10^5	0	70	50	0

Table 2.37 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position five of wheat cv. Monad on Martins medium and Nutrient agar over one sample date at the Pendarvis trial during 1996/97. Sample date was: 5, 19/11/96

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
5	7	2.8×10^3	0	0	0	0

Table 2.38 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position six of wheat cv. Monad on Martins medium and Nutrient agar over two sampling dates at the Pendarvis trial during 1996/97. Sampling dates were: 5, 19/11/96, 6, 2/12/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
5	6.6×10^3	576	0	0	30	10
6	4.2×10^3	2.4×10^{103}	104	0	57	13

Table 2.39 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position seven of wheat cv. Monad on Martins medium and Nutrient agar over four sampling dates at the Pendarvis trial during 1996/97. Sampling dates were: 5, 19/11/96, 6, 2/12/96, 7, 16/12/96, 8, 2/1/97.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
5	8×10^3	83	0	0	0	0
6	2×10^3	1.3×10^3	2×10^3	0	100	0
7	0	0	49	4	0	0
8	0	0	45	13	500	30

Table 2.40 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position eight of wheat cv. Monad on Martins medium and Nutrient agar over two sampling dates at the Pendarvis trial during 1996/97. Sampling dates are:. 5, 19/11/96, 6, 2/12/96

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
5	1×10^3	4.1×10^4	0	0	0	0
6	306	1.7×10^3	0	0	0	0

Table 2.41 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of the flag leaf of wheat cv. Monad after application of fungicides over two sampling times at the Pendarvis trial during 1996/97. Sampling times were: 7, 16/12/96, 8, 2/1/97. Treatments were (1) untreated water control, (2) tebuconazole applied at GS 49, (3) azoxystrobin applied at GS 49, (4) untreated water control, (5) tebuconazole applied at GS 65, (6) azoxystrobin applied at GS 65.

Date	Treatment	Bacterial colonies/gfwt leaf tissue (Martins)	Bacterial colonies/gfwt leaf tissue (NA)	Fungal colonies/gfwt leaf tissue (Martins)	Fungal colonies/gfwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
7	1	13	421	0	0	0	0
	2	76	792	0	0	0	0
	3	85	386	0	0	0	0
	4	0	435	0	0	0	0
	5	51	541	0	0	0	0
	6	168	539	0	0	0	0
8	1	27	237	0	0	0	0
	2	0	912	0	0	0	0
	3	11	709	0	0	0	0
	4	0	899	0	0	0	0
	5	2	676	0	0	0	0
	6	3	50	0	0	0	0

Methven

Table 2.42 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position one of wheat cv. Monad on Martins medium and Nutrient agar over two sampling dates at the Methven trial during 1996/97. Sampling times were: 3, 22/10/96, 4, 5/11/96.

Date	Bacterial colonies/g fwt leaf tissue (Martin)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	544	0	0	12	0
4	0	5.2×10^4	0	0	110	0

Table 2.43 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position two of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Methven trial during 1996/97. Sampling dates were: 3, 22/10/96, 4, 5/11/96, 5, 19/11/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	3×10^3	0	0	0	0
4	0	1.9×10^5	0	2×10^3	4×10^5	0
5	0	6.6×10^5	0	0	5×10^5	0

Table 2.44 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position three of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Methven trial during 1996/97. Sampling dates were: 3, 22/10/96, 4, 5/11/96, 5, 19/11/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	3.3×10^3	0	0	0	0
4	0	200	0	0	0	0
5	0	8.3×10^4	3×10^3	70	6×10^5	0

Table 2.45 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position four of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Methven trial during 1996/97. Sampling dates were: 3, 22/10/96, 4, 5/11/96, 5, 19/11/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
3	0	2.2×10^3	0	0	0	0
4	0	1.2×10^4	0	0	0	0
5	0	1.2×10^3	0	0	0	0

Table 2.46 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position five of wheat cv. Monad on Martins medium and Nutrient agar over four sampling dates at the Methven trial during 1996/97. Sampling dates were: 4, 5/11/96, 5, 19/11/96, 6, 2/12/96, 7, 16/12/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
4	0	101	0	0	0	0
5	4.8×10^3	1.4×10^4	1×10^3	0	200	0
6	5×10^3	9.3×10^3	34	0	332	0
7	0	205	0	0	870	0

Table 2.47 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position six of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Methven trial during 1996/97. Sampling dates were: 5, 19/11/96, 6, 2/12/96, 7, 16/12/96.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
5	0	555	43	0	0	0
6	500	233	0	0	150	0
7	0	94	0	0	780	0

Table 2.48 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of leaf position seven of wheat cv. Monad on Martins medium and Nutrient agar over three sampling dates at the Methven trial during 1996/97. Sampling dates were 6, 2/12/96, 7, 16/12/96, 8, 2/1/97.

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
6	0	233	300	0	0	0
7	0	3	0	0	0	0
8	0	48	0	0	0	0

Table 2.49 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of the flag leaf of wheat cv. Monad on Martins medium and Nutrient agar over one sample date at the Methven trial during 1996/97. Sampling date was 6, 2/12/96,

Date	Bacterial colonies/g fwt leaf tissue (Martins)	Bacterial colonies/g fwt leaf tissue (NA)	Fungal colonies/g fwt leaf tissue (Martins)	Fungal colonies/g fwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
6	240	581	17	0	40	0

Methven

Table 2.50 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of the flag leaf of wheat cv. Monad after application of fungicides over three sampling dates at the Methven trial during 1996/97. Martins medium and nutrient agar were used. Sampling times were: 7, 16/12/96, 8, 2/1/97, 9, 22/1/97. Treatments were (1) untreated water control, (2) tebuconazole applied at GS 49, (3) azoxystrobin applied at GS 49, (4) untreated water control, (5) tebuconazole applied at GS 65, (6) azoxystrobin applied at GS 65.

Date	Treatment	Bacterial colonies/gfwt leaf tissue (Martins)	Bacterial colonies/gfwt leaf tissue (NA)	Fungal colonies/gfwt leaf tissue (Martins)	Fungal colonies/gfwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
7	1	18	3	0	3	0	0
	2	1	0	0	0	0	0
	3	15	0	0	0	0	0
8	1	2	39	0	0	0	0
	2	2	93	0	0	0	0
	3	13	387	0	0	0	0
	4	1	259	0	0	0	0
	5	2	35	0	0	0	0
	6	2	139	0	0	0	0
9	1	0	805	0	0	0	0
	2	0	913	0	0	0	0
	3	0	23	0	0	0	0
	4	0	86	0	0	0	0
	5	0	43	0	0	0	0
	6	0	73	0	0	0	0

Aylesbury

Table 2.51 The numbers of bacterial and fungal colonies per gram fresh weight of leaf tissue present in the phylloplane of the flag leaf of wheat cv. Monad after application of fungicides over two sampling times at the Aylesbury trial during 1996/97. Martins medium and Nutrient agar were used. Sample time was: 7, 16/12/96. Treatments were (1) untreated water control, (2) tebuconazole applied at GS 49, (3) azoxystrobin applied at GS 49, (4) untreated water control, (5) tebuconazole applied at GS 65, (6) azoxystrobin applied at GS 65.

Date	Treatment	Bacterial colonies/gfwt leaf tissue (Martins)	Bacterial colonies/gfwt leaf tissue (NA)	Fungal colonies/gfwt leaf tissue (Martins)	Fungal colonies/gfwt leaf tissue (NA)	Yeast colonies/g fwt leaf tissue (Martins)	Yeast colonies/g fwt leaf tissue (NA)
7	1	14	176	0	0	0	0
	2	3	152	0	0	0	0
	3	0	269	0	0	0	0
	4	2	182	0	0	0	0
	5	0	125	0	0	0	0
	6	0	79	0	0	0	0

2.206 The effect of fungicide application on the duration of green flag leaf area.

The trials at Pendarvis, Highbank, and Aylesbury (1996/97) and Chertsey, Mitcham and Norwood (1995/96) showed no significant increase in the duration of green leaf area after the application of fungicides. The trials at Methven (1995/96 and 1996/97) exhibited effects of fungicide applications. In 1995/96 the rate of senescence of the control was significantly higher than the tebuconazole (187.5gai/ha) and azoxystrobin (250gai/ha) treated plots. The next year at 19/11/96 the later application of azoxystrobin reduced the rate of senescence compared to the two controls, and at 16/12/96 azoxystrobin (late application) reduced the rate of senescence as compared to the control and the early tebuconazole treatment. The "rate of senescence" data for all other leaf positions has not been included as preliminary statistical analysis indicated no significant difference between treatments.

2.207 The effect of Tebuconazole and Azoxystrobin on the germination of conidia

The rate of conidial germination was significantly ($p<0.0005$) higher on the control plates (i.e. no fungicide) than those present on media amended either with the full field rate of tebuconazole (0.19gai/ml) and azoxystrobin (0.25gai/ml), the half field rate of azoxystrobin (0.125gai/ml), and at double the field rate of both azoxystrobin (0.5gai/ml) and tebuconazole (0.38gai/ml). The rate of conidial germination on media amended with half field rate of tebuconazole (0.09gai/ml) was not significantly different from the control. The rate of germination of conidia was lowest on media amended with twice the field rate of azoxystrobin, but there were no statistically significant differences for the level of inhibition of conidial germination between azoxystrobin treatments. The level of inhibition of conidial germination was significantly less on media amended with tebuconazole than media amended with azoxystrobin.

2.208 Effect of Tebuconazole and Azoxystrobin on the growth of *Didymella* in culture

Radial growth of *Didymella* cultures was significantly inhibited by all concentrations of tebuconazole investigated, and by the field rate and double the field rate of azoxystrobin. Half the field rate of azoxystrobin and the control treatment were not significantly different from each other but were significantly different from all the other treatments. This experiment was repeated three times with similar results.

2.209 Yield effects

2.2091 1995/96

The effect of timing of application of fungicide on yield was not significant, so results were pooled therefore reflecting only different fungicide treatments. In all the trials the application of fungicides increased the yield, but the effects of fungicide application were only significant at the Chertsey and the Norwood trials (Table 2.52). Thousand grain weight was also higher in fungicide treated plots in all trials but only by a statistically significant amount ($p<0.05$) at Cherstey and Mitcham (Table 2.52). Yield

was higher in fungicide treated plots than in untreated control plots with the largest yield increase at Chertsey of 18% (Table 2.53). Grain weight was increased by under 5% at Mitcham, Methven and Norwood but at Chertsey the increase was 11.9%.

Table 2.52 The yield (t/ha) and grain weight (mg) of wheat cv. Monad and cv. Endeavour treated with either a water control, tebuconazole or azoxystrobin at four trials sited at Chertsey, Mitcham, Methven and Norwood during the 1995/96 growing season.

Treatment	Yield (t/ha)	Grain weight (mg)
Cherstey		
control	3.6a	27a
tebuconazole	4.2b	30b
azoxystrobin	4.3b	30.4b
Norwood		
control	7.1a	46
tebuconazole	7.4b	47
azoxystrobin	7.3b	47
Mitcham		
control	4.5	34.4a
tebuconazole	4.8	35.4b
azoxystrobin	5.1	36.4b
Methven		
control	7.7	40.1
tebuconazole	8	42.2
azoxystrobin	8.1	42.3

Letters indicate difference ($p<0.05$) as indicated by Duncan' Multiple range test

Table 2.53 The percentage increase in yield and grain weight in wheat cv. Monad and cv. Endeavour compared to the control after the application of either tebuconazole or azoxystrobin (data pooled between fungicides) at the trials sited at Chertsey, Mitcham, Methven and Norwood during the 1995/96 growing season.

Trial	Percentage increase in yield	Percentage increase in grain weight
Chertsey	18	11.9
Norwood	15	2.2
Mitcham	10	4.4
Methven	4.5	5.4

2.2092 1996/97

Yield was not significantly higher in the fungicide treated plots than the control plots however, the yields were always higher in the fungicide treated plots than the untreated controls (Table 2.54). In the Methven trial, azoxystrobin gave an 11% yield increase over the untreated control (Table 2.55). Most of the observable increases in yield came from increases in grain weight. At Pendarvis and Methven the grain weight was higher from azoxystrobin treated plots than from tebuconazole and the untreated controls. When the fungicide data was pooled yield increase compared to the control plots was up to 8.5% and grain weight up to 5.3% (Table 2.55).

Table 2.54 The yield (t/ha) and grain weight (mg) of wheat cv. Monad treated with either a water control, tebuconazole or azoxystrobin at four trials sited at Pendarvis, Aylesbury, Highbank and Methven during the 1996/97 growing season.

Treatment	Yield (t/ha)	Grain weight (mg)
Pendarvis		
control	8.28	48a
tebuconazole	8.46	49.2a
azoxystrobin	8.66	49.6b
Aylesbury		
control	5.1	45.5a
tebuconazole	5.2	46.5a
azoxystrobin	5.33	47.4a
Highbank		
control	8.5	45.5a
tebuconazole	8.7	46.5a
azoxystrobin	8.7	47.4a
Methven		
control	6.5	47.6a
tebuconazole	6.9	49.1a
azoxystrobin	7.2	51.1b

Letters indicate differences ($p<0.05$) as indicated by Tukey's Multiple range test.

Table 2.55 The percentage increase in yield and grain weight in wheat cv. Monad compared to the control after the application either tebuconazole or azoxystrobin (data pooled between fungicides) at the trials sited at Pendarvis, Aylesbury, Highbank, and Methven during the 1996/97 growing season.

Trial	Percentage increase in yield	Percentage increase in grain weight
Pendarvis	4.6	2.9
Aylesbury	4.5	3.2
Highbank	2.4	3.2
Methven	11	5.3

2.30 Discussion

Didymella appears to enter spring wheat crops about a week after the first leaf unfolds. It is then either transmitted systemically through the host tissue, or infection occurs of the other leaves by the way of ascospores or conidia. *Didymella* is not consistently isolated from the leaves throughout the growing season, presumably due to the subcuticular intramural location of its hyphae which renders it very sensitive to environmental conditions.

Weather data was obtained from the National Institute of Water and Atmosphere (NIWA) weather stations at Lincoln Broadfield, Ashburton Township and Darfield. Very little correlation was obtained between the levels of *Didymella* cultured from surface sterilised green leaf tissue and environmental conditions. This could have been due to the fact they were only monthly summaries and did not take into account the daily fluctuations in the weather conditions which may or may not have affected the fungus.

The 1995/96 season was relatively cool, but rainfall was variable.The 1996/97 season had similar rainfall levels although slightly higher at the Lincoln Broadfield and Darfield sites but lower than the previous year at the Ashburton site. The maximum and minimum temperatures were very similar.

There was a higher level of *Didymella* sporulation during the 1996/97 growing season than during the 1995/96 season. Although no correlation between *Didymella* levels and the weather data could be obtained it is possible that the environmental conditions, for example, the slightly higher rainfall during the 1996/97 season was influencing the levels of *Didymella.* For both years the levels of *Didymella* were higher at the Methven sites than the other trial sites. There are few factors that differed between these trials, grasses at crop margins were controlled to a similar level and they all received similar agronomic treatment. One of the only possible reasons for the higher levels is geographic location and thus a slightly different set of environmental conditions.

Riesen (1987) had suggested that the dry windy conditions of the Canterbury plains is the cause of fluctuations in levels of *D. phleina* in barley and in view of its subcuticular intramural location within the leaf, hot dry conditions may eliminate the fungus from the host leaf tissue. The weather data also did not correlate well with *Sporobolomyces* levels or bacterial numbers. To investigate the effect of weather conditions on the amount of *Didymella* present within a crop, a weather station within each crop would be needed with daily readings taken of temperature, relative humidity, wind direction and rainfall.

In Switzerland, more than 200 species of fungi have been isolated from wheat leaves, whereas in Canterbury only 13 were isolated from barley, probably due to the environmental conditions and the geographic isolation of New Zealand leading to lower species diversity (Riesen 1987). In the present study only 10 species were isolated from wheat over the two years that leaf plating was carried out. It remains unknown what causes the fluctuating numbers of endophytic fungi; it may be either the changing microenvironment of the leaf, changing interactions between species on the leaf surface, changing environmental conditions or by changing airspora.

Di Menna (1959) found no correlation between humidity, temperature, rainfall and the number of yeast colonies present on leaves. She thought it was probably a

combination of the above factors. In the present study no correlation could be obtained between yeast numbers and the environmental conditions.

2.31 Indirect method of enumeration of phylloplane micro-organisms

Bacterial and yeast numbers tended to increase as the leaf material aged. This has also been found by other researchers (Di Menna 1959). Di Menna (1959) believed the differences in the number of colonies was due to availability of different levels of nutrients. Filamentous fungal numbers also increased. The use of leaf washing to investigate fungal population densities was relatively unsuccessful as bacteria tended to spread over the plates before fungal numbers could be enumerated properly. In an attempt to rectify this problem Martins medium was used instead of PDA. This medium inhibited bacterial growth long enough for the fungal numbers to be enumerated, however the fungi commonly isolated were relatively fast growing and tended to overgrow slower isolates resulting in a less than clear picture of the phylloplane inhabitants. The bacteria isolated tended to be Gram negative rods and the fungi were *Alternaria* spp. and *Penicillium* spp. Fungal levels fluctuated throughout the growing season. Di Menna (1959) found the leaf washing technique was very effective at removing yeast colonies, however, the present study found the spore fall method more effective. Yeast colonies isolated using the leaf washing method tended to get overgrown by bacteria quickly. Other researchers (Di Menna 1959, Andrews *et al.* 1980) have not had similar problems. The use of antibiotic amended media would have allowed for greater investigation of fungal species but would not have overcome the problem of fast growing *Alternaria*. The only way that would have overcome this problem was the subculturing of every fungal isolate within a two days of plating. This was not logistically possible.

Direct techniques for the evaluation of phylloplane organisms were also evaluated; these being leaf impressions, high humidity and near-UV light and a leaf imprinting technique. The leaf impression technique was extremely effective for the evaluation of *Penicillium* numbers and in later samples the *Sporobolomyces* populations, however, due to the fast growing nature of *Penicillium* and its ability to

produce copious spores it was difficult to investigate any other fungi that were present. The topography of the leaf could also not be investigated using this method which reduced its usefulness. It did not allow the positions of microorganisms on the leaf surface to be investigated, for example, Last (1955a) stated that *Sporobolomyces* populations were larger at the distal end.

The leaf impression technique used in this study has several disadvantages in that the natural arrangement of micro-organisms may have been disturbed and it may also may be difficult to stain the micro-organisms differentially (Dickinson *et al.* 1974). Soil on the leaves was a major problem for this method as it obscured many spores. Due to irrigation and rainfall, soil particles were observed to be present on all leaf positions examined with the most particles present on leaves one to four. However it allowed the identification of several pathogen species for example *S. nodorum* and *S. tritici* and also showed that they were relatively uncommon inhabitants of the phylloplane. The most common inhabitants were yeast species and these were present over the entire leaf surface. This result was similar to that of Last (1955b) who found that *Sporobolomyces* levels were relatively low until the leaves were half way through their lifespan and the maximum level of *Sporobolomyces* was reached after the leaf died. The distal part of the leaf had more colonies present than did the proximal but the upper and lower surfaces of the aging leaves carried similar numbers of colonies. In the present study no effect of leaf orientation could be found but the distal portion of the leaf had more *Sporobolomyces* colonies.

Alternaria was the only fungus identified on leaves placed under near-UV light with high humidity and this technique did not allow for enumeration of the organisms present. The embedded leaves reduced the numbers of bacteria isolated but it was very difficult to enumerate the number of colonies.

The suspended leaf technique for the enumeration of the *Sporobolomyces* populations showed there was no effect of fungicide treatment or of leaf orientation on *Sporobolomyces* levels in either the Pendarvis, the Methven or the Winchester trial.

Pennycook and Newhook (1978) used both the spore fall method and the macerating leaves and then performing a dilution plating method to investigate the accuracy of the different methods. They found no consistent relationship between the results of the two methods indicating that the population size and the number of ballistospores discharged are not proportional. They stated that the spore fall data should not be used as an estimate of population density. In the present study the data was not used as an estimate of the actual population density. It was used as a measure of the sporulation of *Sporobolomyces* between the fungicide treated leaves as to the effects of fungicides on the density of *Sporobolomyces* populations. The effect of time of day on ballistospore discharge was avoided by using a 24 h exposure period (Pennycock and Newhook 1974). Bashi and Fokkema (1976) found that ballistospore production can be radically altered even under seemingly identical environmental conditions. This was found in the current study as levels on individual leaves varied greatly.

The results of the enumeration of phylloplane organisms emphasised the lack of hyphal growth on newly expanded leaves which agreed with the data obtained by Dickinson (1967) for phylloplane activity on pea leaves. Unlike the *Pisum* leaves in wheat there appeared to be no extensive fungal activity that began at senescence. The leaves were not rapidly colonised by mycelial fungi, although several saprophytic species were observed on cleared leaves. The leaf washing method was relatively inefficient at allowing the isolation of phyllosphere fungi as the peptone seemed act as a buffer for the bacteria cells but not the fungal cells allowing greater survival of bacteria. 'Tween' 20 may have been more appropriate to use so as to increase the removal of leaf surface propagules (Dickinson 1967). The moist chamber treatment indicated the presence of potential fungal colonists but *Alternaria* was commonly isolated and this tended to mask the development of other fungi. Bacterial and yeast levels were not monitored by this method.

The techniques used when considered together showed that there was a reduction in phyllosphere bacterial levels after both fungicide and water control applications. Bacterial levels were not significantly lower on fungicide treated leaves compared to the

control leaves. During the 1995/96 growing season bacterial levels increased as the wheat plants aged but during the 1996/97 season levels reduced after the application of both the fungicides and the water control. Levels never returned to pre application levels within the sampling time. Other abiotic and biotic factors that were not identified must have caused the reductions in bacterial microflora. Levels of yeast and fungi fluctuated regardless of treatment whether it was fungicide or water. Within the leaf laminar levels of bacteria and fungi were not greatly different from pre-fungicide application levels during the 1996/97 field season. During the 1995/96 growing season the only fungus that was controlled by fungicide application was *Didymella* spp and the fungicide was azoxystrobin. The level of sensitivity of the phyllosphere fungi isolated in this study to the fungicides used is not known. It appears that phyllosphere organisms are either not sensitive or the levels of fungicides drop off rapidly allowing the phyllosphere organisms to multiply again.

Work by other researchers has shown that generally fungicide application greatly affects the microflora of flag leaves and ears reducing fungal levels for between two and six weeks (Hill and Lacey 1983, Magan and Lacey 1986). Hill and Lacey (1983) confirmed the findings of Edington *et al.* (1971 in Hill and Lacey 1983) that *Alternaria* was tolerant to benomyl treatment and levels were even increased. They suggested that the increased levels were due to *Alternaria* filling the niches previously occupied by *Cladosporium* which was sensitive to benomyl application. None of the fungal species isolated during the course of the current study appeared be affected by fungicide application. Levels of all the species fluctuated regardless of either fungicide or water application. As Dickinson (1973) found *Sporobolomyces* levels were not affected greatly by the application of fungicides

2.32 Antagonism

Didymella was overgrown in plate tests by *Phoma* spp., *Fusarium* spp., and *Helminthosporium* spp. *Sporobolomyces* was the only isolate not to reduce or completely inhibit the growth of *Didymella*. The majority of bacterial isolates obtained from the leaf washings, when plated against *Didymella,* caused slight to complete

inhibition of growth. From these plate tests it appears that *Didymella* does not play a phytoprotection role.

The seeds that had been infected with *Didymella* on plate tests grew into healthy plants. The germination rate of the seeds was normal and the leaves from these plants were plated out to see if *Didymella* was present within the leaf tissue. No *Didymella* was cultured showing that for the *Didymella* isolates used and for wheat, *Didymella* was not seed borne or transmissible via this route. This has also been found for *Ascochyta rabiei* on chickpea which is not be transmitted from the seed to the foliar plant part unless via a nonsystemic route (Dey and Singh 1994)

Disease levels in the trials during both the 1995/96 and the 1996/97 growing seasons were low. During both growing seasons, powdery mildew and stripe rust were present in the trials. Few scorch symptoms of either *Septoria* spp. or *Didymella* spp. were found on wheat plants in the trials.

2.33 The effects of fungicides on *Didymella* levels

Didymella was not isolated from leaves treated with azoxystrobin during the 1995/96 field season. The levels of *Didymella* isolated from tebuconazole treated wheat flag leaves were in some cases higher than the control, although this result was not statistically significant. The levels of *Didymella* on leaves treated with azoxystrobin were significantly lower than the control and the tebuconazole treated leaves during the 1995/96 wheat growing season. There was no effect of the timing of the fungicide sprays on the levels of *Didymella* isolated from treated flag leaves. This pattern changed in the 1996/97 growing season; *Didymella* was cultured from all treatments and there was no statistically significant effect of fungicide treatment versus the untreated control. The reason for this sudden change in the effectiveness of azoxystrobin remains unknown, however, a similar result was found in culture plate tests, azoxystrobin only reduced the level of growth compared to the untreated control. In culture the

germination of conidia was inhibited and no culture growing on azoxystrobin-amended media produced pycnidia. On the other hand, growth was reduced markedly on media amended with tebuconazole but the germination of conidia was not.

On senesced leaf material, the fungus sporulated to a greater extent on the tebuconazole treated leaves than on the azoxystrobin treated leaves, which seems to indicate a similar result to that obtained from laboratory tests. There were no significant differences in the bacterial levels or the levels of such fungi as *Alternaria* and *Cladosporium* in fungicide treated leaves versus the controls. *Fusarium* spp., *S. nodorum* and *Helminthosporium* spp., were cultured from leaf material very infrequently and the effects of the fungicides on these fungi could not be established.

Godwin *et al.* (1994) investigated the effects of preventive applications of azoxystrobin on *P. recondita*, *S. tritici* and *S. nodorum* and like *Didymella*, spore germination was inhibited.

Broad spectrum fungicides often affect the saprophytic microflora succession on leaf tissue (Lacey *et al.* 1986 in Magan and Lacey 1986). Due to the variability in fungal population size, reductions in numbers could not be attributed to the fungicide sprays in the current study. Increases in crop yields have been found in crops that have been treated with broad spectrum fungicide in absence of any or low, disease pressure (Dickinson and Walpole 1975, Mappes and Hampel 1977, Cook 1980, Priestly 1981). In this study the disease pressure was low and yield increases were found up to 18% during the 1995/96 season and up to 8.5% during the 1996/97 season. Grain weight also increased in fungicide treated plots and most of the yield increase came from increases in grain weight. Increase in yield and grain weight were a substantially less during the 1996/97 season than in the 1995/96 growing season. This may be due to physiological reasons or alternatively due to the higher levels of *Didymella* spp. found during the 1996/97 season. The actual role that *Didymella* plays in yield reduction still remains elusive. The lack of control offered by azoxystrobin during the 1996/97 growing season makes any generalisation about *Didymella's* role difficult. Tebuconazole did not

eliminate *Didymella,* in fact, during the 1995/96 season, it appeared to encourage, it and the yields in these plots were still higher than the untreated control indicating that *Didymella* spp. played little or no role in yield reduction.

The duration of green leaf area was significantly longer at the two Methven trials after the application of azoxystrobin compared to the untreated control. This increased time of green leaf area may explain some of the yield increase and there are two possible reasons for it. The first is that the fungicide had some physiological effect on the host in these trials, or saprophytes were accelerating the rate of senescence in untreated plots.

This observed yield increase may be caused by weakly saprophytic fungi such as *Alternaria* and *Cladosporium* hastening senescence and reducing the period of grain fill (Dickinson 1981, Priestly 1981) or alternatively it may be due to the hormone effects of the fungicide. The increase in yield may not be large enough to warrant the application of fungicides to control saprophytes. In this study the levels of saprophytes for example *Alternaria, Cladosporium* within the leaf laminar, were not reduced compared to levels on untreated leaves so it is unlikely that the increases in yield were due to the control of these fungi. Their role however cannot be discounted without further investigation.

It is speculated that saprophytes may affect host plants in two different ways (Tolstrup 1984 in Jachmann and Fehrmann 1989, Tolstrup and Smedgaard -Petersen 1984 in Jachmann and Fehrmann 1989). The activation of defence mechanisms after inoculation may lead to a reduction in plant yield. A weak parasite may also cause accelerated senescence. Due to the complicated nature of the interaction between the host and the saprophytes it is probable that the actual mode is a combination of both possibilities.

Riesen (1987) found that sprays of propiconazole reduced the level of *Alternaria* and *Cladosporium* spp. within a barley crop. Pugh and Buckley (1971) suggested that *Cladosporium* may influence senescence of wheat plants by either direct penetration of the host tissue or by inducing a resistance or defence mechanism.

In 1955, Last (in Bashi and Fokkema 1977) suggested that nutrient competition in the phyllosphere may result in a reduction in the superficial development of the pathogen due to a reduction in infection. Other factors such as continuous rain may also affect the densities of microorganisms (Fokkema 1971). After cereal leaves were half way through their life span, the development of *Sporobolomyces roseus* increases (Bashi and Fokkema 1977). In the present study the levels of *Sporobolomyces* increased as the leaves aged thus providing increased levels of nutrient competition which may have reduced *Didymella* levels.

In the case of *Didymella* in this study, the *Sporobolomyces* populations were not affected by the application of fungicides, therefore, it is unlikely that the increases seen during the 1995/96 growing season in the *Didymella* levels on tebuconazole treated leaves was due to a reduction in *Sporobolomyces* levels. *Sporobolomyces* was also the only species not to inhibit the growth of *Didymella* in plate tests in the laboratory.

Trials have established the increase in yield gained by spraying fungicides in the absence of disease pressure. In studies carried out by Tolstrup (1984 in Jachmann and Fehrmann 1989) and Tolstrup and Smedgaard-Petersen (1984 in Jachmann and Fehrmann 1989) of the chlorophyll content and the amount of senescence in oats and peas treated with different fungicides (captafol triadimefon, propiconazole and prochloraz) it was found the yield of the oats had increased by 10-16% in the plots treated with captafol and propiconazole. However, prochloraz and triadimefon had no significant effect on yield. From this result, the effect of the fungicide on the physiology of the host cannot be discounted, however, it seems likely that the reduction of the saprophytes reduced the number of plant defence reactions, thus making more energy available for grain production. In this thesis the application of fungicides increased yield but the levels of saprophytes were not greatly reduced. From this result it seems likely that the fungicides were having a physiological effect on the host plant. It is not known from this study what reduction in the level of saprophytes is needed before yield is increased. Perhaps the level of reduction of saprophytes obtained in this study was great

enough to allow yield to increase due to a reduction in the number of plant defence reactions.

Foliar fungicides were investigated by Entz *et al.* (1990) for their effects on grain yield, kernel size and seed size distribution in wheat and barley. Wheat trials were sprayed with Tilt between growth stages 49-59. Diseases that were present were leaf rust, tan spot and *Septoria* leaf spot which were controlled by Tilt applications. Yields were significantly increased as was the prevalence of larger kernels. The number of large kernels increased, even under low disease pressure . Kernel size is an important factor in increasing seed vigour (Kaufman 1984 in Entz *et al.*).

No statistically significant increases in yield or grain weight could be established in the majority of the trials reported here as a result of fungicide treatment, except for Chertsey and Norwood. There was however, an increase of up to 11% in grain weight following the application of fungicides and smaller increases in yield. The levels the endophytes and saprophytes investigated dropped until around 14 days after application when levels rose to pre fungicide application levels. The levels of fungi within the leaf laminar did not drop as much as the levels in the phylloplane.

Other researchers have found that late season applications of fungicides increase kernel size in wheat (Spiertz 1973, Cook 1980, Priestly and Bayles 1982, Lorenz and Cothran 1989) and barley (Priestly and Bayles 1982, Hill and Lacey 1983, Tolstrup and Smedgaard-Peterson 1984 in Jachmann and Fehrmann 1989, Riesen and Close 1987). In the present study, there was no effect of timing of spray application but the application of fungicides increased the grain weight compared to the untreated controls during both growing seasons. Cook (1980) suggested that carbohydrates usually translocated to developing kernels may be utilised by foliar plant pathogens and that the fungicides applied would stop this happening.

2.34 Field survey

The surveys carried out by Cromey *et al.* 1994a, Cromey *et al.* 1994b, and Cromey and Mace (1995) indicated that *Didymella* spp. are widespread in both the Canterbury region and other wheat growing areas, for example, the lower North Island. Cromey *et al.* 1994b identified only five pathogens in sampled crops, *Didymella* spp. *S. nodorum*, *Puccinia striiformis*, *Mycosphaerella graminicola* and *Ascochyta. Didymella* was present on 91% of leaves and the mean leaf area affected was 23%. The next most common pathogen was *S. nodorum* at 86% and 1.8% of leaf area affected. The other pathogens affected the leaves to a lesser extent. In this study, two field surveys were carried out, one in 1996 and the other during 1997. In the 1996 survey of Canterbury, of 14 wheat crops surveyed, 70% were infected and pseudothecia covered 2.8% of the leaf area. Twenty percent of barley crops were infected, with 1% of the leaf area affected. During 1997 *Didymella* was found to be the most widespread fungus on wheat, present in 97% of crops surveyed, and affecting 11% of the leaf area. In these two surveys *Ascochyta* and *Didymella* results were combined as the pycnidia and pseudothecia were intermingled on the leaf tissue. *S. nodorum* was found in 18% of crops covering 2% of the leaf area and *S. tritici* affected 14.3% of crops and covered 0.83% of the leaf area. The levels of *S. nodorum* and *S. tritici* found in the current study were a lot lower than those found by Cromey *et al.* (1994b). The reasons for this are unknown, possibly weather conditions were not conducive to these two pathogens or disease forecasting allowed improved timing of fungicide application.

2.35 Resistance

No cultivars resistant to *Didymella* were found in the Arable Cultivar Evaluation trials. The trial site had a statistically significant effect on *Didymella* levels as did year. Levels of *Didymella* were higher during the 1996/97 growing season than the 1995/96 wheat growing season. Possible reasons for this include different environmental conditions and the amount of inoculum, that is the amount of control of grasses at the sides of the fields. This finding was similar to that of Paul in 1982 when he investigated 48 winter wheat cultivars and found no resistant cultivars. Cromey *et al.* (1994a) found a difference between cultivars in the level of sporulation on leaf material, however,

Cromey (pers. comm. 1995) suggested that this result may have been due to leaf material being collected when plants were different relative ages. The method used in the current study allowed for the removal of bias associated with collecting plants of different relative ages. Cultivars could thus be assessed taking into account both leaf age and the time of senescence.

In conclusion it appears that *Didymella* does not play a role in accelerating leaf senescence and reducing yield under the environmental conditions experienced during the course of this study. It is commonly found sporulating on senesced leaf tissue of wheat, barley, oats, triticale and grasses present at crop margins. No cultivar resistant to *Didymella* could be identified. Phyllosphere bacterial and fungal levels did not appear to be influenced by the application of fungicides.

3.0 INFECTION PROCESSES OF *DIDYMELLA* SPECIES

3.01 Introduction

There are doubts as to whether *Didymella* is considered a pathogen as opposed to an endophyte, although it must infect its host for its continued survival. This requires that *Didymella* spores must land on a susceptible plant surface and conditions must be suitable for the spores to germinate, form germ tubes, then appressoria and finally penetrate the host's epidermal cells. Many conditions, both abiotic and biotic affect this process.

3.02 Environmental effects

There are many environmental effects that may influence infection of a host by a plant pathogenic or an endophytic fungus on host leaf tissue. These are the amount and duration of moisture present on the leaf surface, the amount and quality of light and the temperature surrounding the leaf tissue. Many pathogenic fungi require different optimum environmental conditions at different stages of the infection cycle. The specific needs at these different stages provide only a short period of time that the infection can take place therefore the more successful pathogens will infect the host quickly. After infection the pathogen is less vulnerable within the host as the environmental conditions remain relatively constant (Tarr 1972).

3.03 Humidity

For micro-organisms, atmospheric humidity plays a very important role. Often it is the main component for the infection of the host plant by pathogenic fungi. It also controls the amount of free moisture, thus having an indirect effect on plant pathogenic fungi (Harrison *et al.* 1994). Different stages in the life cycle of fungi may have different humidity requirements. Once infection has occurred and the pathogen is established, atmospheric humidity plays a very small role, if any, because the plant, as long as it is not wilted, remains at or near saturation (Rose 1966 in Harrison *et al.* 1994).

The majority of diseases are encouraged by high humidity. There are, of course, exceptions to this, one being *Uromyces fabae* (brown rust of field bean) which causes

severe disease during dry summers in Scotland (Harrison *et al.* 1994). Germination of conidia is generally the time when the highest humidity is required. It is very difficult to measure and control the humidity at the plant surface. Transpiration plays a major role in the difficulties of maintaining constant humidity at the leaf surface in laboratory experiments. Saturated salt solutions are commonly used to control humidity because they have the capability to absorb excess water vapour if the salt solution is saturated and some solute is present (*e.g.* Ward and Manners 1974 in Harrison *et al.* 1994, Harrison 1983a in Harrison *et al.* 1994, Shaw 1986 in Harrison *et al.* 1994, Vanniasingham and Gilligan 1989 in Harrison *et al.* 1994, Everts and Lacy 1990 in Harrison *et al.* 1994). Other techniques have been used. Hammond *et al.*, (1985) lined plastic propagator pots with absorbent paper that was wetted every 12 h to maintain near saturation point humidity.

3.04 Temperature

Temperature also plays a major role in the infection processes of many fungi. Fungal spores generally germinate over a wide temperature range with the optimum being around 15-25^{0}C (Tarr 1972). Optimum temperatures for germination of spores may not be the same as the temperature required for either the growth of germ tubes or for the production of appressoria (Tarr 1972). Skoropad (1967) found that *Colletotrichum graminicola* formed appressoria at temperatures ranging from 15-35^{0}C however it cannot penetrate barley leaves unless the range is 25-35^{0}C. *Septoria tritici* and *S. nodorum* require a period of high humidity and temperatures of 12-17^{0}C after inoculation for the infection of wheat (Holmes and Colhoun 1974).

3.05 Exudates on the plant surface

As plant leaf tissue ages, greater cell leakage can occur and metabolites from other micro-organisms may be present on the leaf surface. These compounds can either have a positive or negative effect on germination of fungal spores (Tarr 1972). Two examples of this can be seen in conidia of *Colletotrichum musae* and *Colletotrichum acutatum,* where the presence of nutrients enhances the germination rate, however

nutrients present in even small quantities may interfere with the production of appressoria (Mercer *et al.* 1971 in Bailey *et al.* 1992).

3.06 Age of leaf tissue

Resistance to disease may change over the life cycle of a plant and many factors are involved in this. The effectiveness of the plant's resistance mechanisms change as the plant ages. In the young plant, phytoalexins may be produced in greater quantities or more readily whereas the older plant may have had biochemical changes taking place within its tissues which make it either more or less suitable for the pathogen to colonise (Tarr 1972). Elmer and Ferrandino (1995) investigated the effects of leaf age on infection of tomato by *S. lycopersici*. When low inoculum (10^1-10^2 spores/cm^2 of leaf) densities were used, young and old leaves were equally susceptible but older leaves were more susceptible when high inoculum densities were used. Carver and Adaigbe (1990) found that fewer conidia of *Erysiphe graminis* germinated on seedling leaves versus adult leaves of wheat.

3.07 Biotic factors

Much research has been directed towards the interactions between micro-organisms in the phylloplane (Dickson 1973, Dickinson and Walpole 1975, Fehrmann *et al.* 1978, Priestley 1981 and Priestley and Bayles 1982). Synergistic and antagonistic relationships may exist between the micro-organisms that make up the phyllosphere, including plant pathogens if only fleetingly. The application of fungicides to control crop diseases may actually be a double-edged sword. It may control the pathogen in question, but it may also kill or limit the number of phylloplane inhabitants, which may in turn control another potential pathogen. Biotic factors are covered in greater detail in chapter 2.

3.08 Infection Processes of *Didymella exitialis*

Only three studies have investigated the infection of wheat by *Didymella* spp., and all these experiments gave similar results. Ahrens and Schöpfer (1983) found that plants inoculated with a spore suspension resulted in only a slight halo, which could not

be observed with the naked eye. Cromey *et al.* (1994) found that plants developed scorch symptoms on leaves two weeks after inoculation with a conidial spore suspension and lesions sporulated when detached and placed under near-UV light.

It is known from Mace (1994) that *Ascochyta* conidia germinate at between 0-12 h after inoculation and form appressoria between 48-72 h under conditions of 18^0C and near 100% humidity. There appeared to be no orientation of the germ tube in relation to the topography of the leaf surface. Penetration of the leaf surface was direct. Initially hyphal growth was subcuticular, intramural and the cuticle was stretched over the hyphae and the cell wall showed only localised disruption. Four days after inoculation the epidermal cells had intracellular hyphae present and in all cases the cells exhibited cellular disorganisation as a result of infection. Few scorch symptoms developed on inoculated leaves.

Parasite - host interaction where the fungus initially establishes a subcuticular relationship in the host leaf before embarking on a more complete invasion are uncommon. Included amongst pathogens that have a subcuticular intramural stage is *Rhynchosporium secalis* which causes scald of barley (Jones and Ayres 1974). This fungus penetrates the host directly and the hyphae then grow along a region of the cuticle that is rich in pectic substances. The epidermal cells show localised disruption and the cuticle remains intact, apart from some localised stretching over the top of hyphae. As the infection progresses the epidermal cells collapse and degenerative changes start to take place in the chloroplasts (Jones and Ayres 1974).

3.09 Aims

The aim of this section of work was to identify the conditions that would lead to initiation of leaf scorching and thus give an indication of the conditions required in the field for the initiation of disease or the endophytic or latent state. Different temperatures and humidities were trialed to examine the effects on spore germination, the formation of appressoria and the actual penetration of the host. To predict which conditions were

optimal the following indicators were used; the percentage germination of spores on the leaf surface and the percentage production appressoria on inoculated leaves

3.10 Materials and Methods - Investigation of the infection processes of *Didymella* species on wheat cv. Monad

3.101 Preparation of spore suspension

Sixteen day cultures of the *Ascochyta* anamorphs of *Didymella* spp. isolated from wheat cv. Otane were used to prepare spore suspensions (Appendix 6). The cultures were flooded with sterile deionised water containing 500 µg/l of 'Tween 20' and the agar surface was then scraped with a sterile microscope slide. The suspensions were then filtered through sterile muslin and the resulting suspensions adjusted to approximately 50,000 spores/ml using a haemocytometer. A drop of spore suspension was pipetted on to each of two PDA plates per isolate to check spore germination. The plates were then incubated at 20^0C with a 12 h photoperiod. To prevent the loss of pathogenicity isolates were subcultured only once on to PDA.

3.102 Preparation of Host Plants

Wheat cv. Monad plants were grown in potting mix in 15 cm pots with four plants per pot on a glasshouse bench at 15^0C with a light cycle of 12 h light and 12 h dark. Plants at GS 39 (Zadoks *et al.* 1974) were prepared for inoculation with spore suspension in the following manner:

(1) Plastic Petri dishes with two holes at opposite ends, large enough for the wheat leaves to be threaded through, were positioned adjacent to pots at leaf height.

(2) Whatman No 3 filter paper, 9 cm in diameter was moistened using sterile deionised water and placed in the dishes to provide a high relative humidity.

(3) The selected leaves were then placed through the holes of the dishes in either the abaxial or the adaxial orientation.

3.103 Plant inoculation

When the plants had reached GS 39 they were inoculated with the above described spore suspension. Approximately 24 µl spore suspension was used to inoculate each of the selected leaves in three places using a sterile Pasteur pipette. Control leaves were inoculated with 24 µl sterile deionised water and 500 µg/l 'Tween' 20. The plants were maintained on a glasshouse bench at approximately 18 - 20^{O}C for a further four days with natural light. The experiment was set up as a randomised complete block. The effects of leaf age were also studied. This was achieved by selecting leaves that were 7, 14, 21, 28 days old and inoculating them using the method described above.

3.104 Examination of infected leaf tissue

One leaf was detached per leaf position from inoculated plants and cleared of chlorophyll, 12, 16, 24, 48 and 72 h after inoculation. One control leaf was also detached per plant. The leaves were placed inoculated side up at the top of a piece of Whatman No 3 (11 cm diameter) filter paper at 45^{0} from the horizontal in a closed container. The 45^{0} angle was achieved by resting a glass plate (10cm by 10cm) in a glass Petri dish and the top of the plate was supported by a plastic well containing the leaf clearing solution. The top of the filter paper was placed inside the well, which contained a clearing solution that consisted of a 75:25 v/v of ethanol and chloroform and 0.15% trichloroacetic acid (Wolf and Fric 1981). The leaves were cleared over a period of four days, during which time the solution was changed frequently.

Once the chlorophyll was removed, the leaf pieces were transferred to an identical closed container with a well containing staining solution. The stain used was protein specific, containing one volume of 15% trichloroacetic acid in water and one volume of 0.6% Coomassie Brilliant Blue in methanol (Wolf and Fric 1981). Leaf pieces were left overnight at room temperature for staining. After clearing and staining, the leaves were placed on glycerol-soaked filter paper, which lined the bottom of Petri dishes, which were then sealed shut using cling film (Glad wrap™). The cleared leaves were mounted in 50% lactic acid and 50% glycerol on a microscope slide and examined

under an Olympus BH2 compound microscope to determine the percentages of germinated conidia and formation of appressoria.

On the fourth day after inoculation, four leaves per isolate and four control leaves were detached. These were placed in glass Petri dishes containing Whatman No 3 filter paper that had been moistened with sterile deionised water. The glass Petri dishes were then placed under near - UV light and checked periodically for the development of pseudothecia and pycnidia.

3.105 Optimisation of infection conditions

Four isolates (Appendix 6) were plated on to PDA (three plates per isolate) from long term storage and grown at 18-20^{0}C under near - UV light for 14 days. After 14 days, spore suspensions were prepared as before. Wheat cv. Monad seeds were planted in 9 cm pots (four seeds/pot) and plants were inoculated when four leaves were fully emerged.

Three sticker solutions were trialed to see if these worked in holding the spore suspension on the leaf material for the time required for germination of conidia: gelatin (0.5%); agar 0.1-0.2%; and 5g/l methyl cellulose, 10g/l glycerol and 0.5g/ 'Tween' 20 (Lancashire and Jones 1985).

The treatments were: 1/ control, water inoculation no spores
2/ spores, 'Tween' 20 and water
3/ agar , 'Tween' 20 and spore suspension
4/ gelatin, 'Tween 20', and spore suspension
5/ methyl cellulose + glycerol + 'Tween' 20 and spore suspension

The sticker solutions were added separately to 10 ml of the spore suspensions and plants were inoculated using either 20µl of spore suspension placed on the leaf surface or an atomiser (delivering 0.25ml/plant). Each spore suspension containing the sticker solution was plated on to PDA plates (3 replicates/isolate). Spore suspensions with no sticker solutions were also plated onto water agar and the plates were incubated at 20^{0}C. Germination of spores was examined under the compound microscope at 2, 4,

6 and 8 h after plating. Germination was recorded as having occurred when the length of the germ tube was as great as the width of the spore (Manners and Hossain 1963 in Carver and Adaigbe 1990)

The experiment was set up as a randomised complete block design. Plants were placed under a humidity chamber, which consisted of a wooden frame covered with clear polythene that was placed over the pots. The pots were sitting in a metal tray filled with water in a glasshouse at 20^0C. The plants remained covered for 96 h after inoculation and every two h after inoculation the plants were misted with water to maintain a high humidity. Plants were examined for a two-week period for the development of symptoms and after two weeks, two leaves per plant were removed and surface sterilised in 0.8% sodium hypochlorite and sterile distilled water. The leaves were then cut into 1cm^2 segments, plated on to PDA and incubated at 20^0C with 12 h light and 12 h dark. Three days later, the plates were examined and number and identity of any fungi present was recorded. Any leaf scorch observed on the surface sterilised segments was assessed using the standard leaf area diagrams for *Septoria* leaf blotch (James 1971).

After carrying out the above experiments in duplicate, a further experiment was carried out to assess whether or not detached leaves gave similar results to attached leaves and also to examine the effects of kinetin on the germination of conidiospores.

3.106 Comparison of infection processes on attached versus detached leaves, inoculum effects and the effects of kinetin

Wheat plants cv. Monad were grown in 9 cm pots as above. Spore suspensions were prepared as above. The whole plant study was set up as above. Leaves of the same age were removed from the plants and cut up into two cm segments. These leaves were placed in Petri dishes lined with Whatman no 4 (9cm diameter) filter paper. The filter paper was either soaked with sterile deionised water or sterile deionised water plus 0.02g/l kinetin. Twenty µl of the spore suspension with added sticker solution was pipetted onto the leaves. Twenty µl of spore suspension and kinetin solution were

pipetted on to water agar plates with three replicates for each isolate. Control plates were incubated with spore suspension only and plates were incubated at 20^0C for 48 h. The plates were then removed at two hourly intervals until 10 h after inoculation and were examined under a compound microscope and the percentage germination was recorded. Germination was defined as above in section 3.25. The results were analysed using ANOVA. The experiment was set up as a randomised complete block.

Leaf pieces were removed from the plates or cut from the plant every two h for 12 hours and then every four h until 48 h after inoculation. The leaf pieces were then cleared and stained using the method of Wolf and Fric (1981). After clearing and staining, the leaf pieces were mounted in 50% lactic acid and 50% glycerol and examined under a compound microscope and the percentage germination of spores and the percentage formation of appressoria were recorded for each treatment.

A comparison was made between the use of mycelial blocks placed on leaf material and spore suspension as used previously as inoculum. Plants were grown and spore suspensions prepared as above. Using a no. 2 cork borer discs of mycelia were removed from the edge of an actively growing culture. Discs from cultures of three isolates were placed on wheat leaves. The experiment was set up as a randomised complete block. The other plants were inoculated with spore suspensions of the same three isolates. Humidity chambers used above were placed over the plants and the plants were misted every two h after inoculation. After 96 h the humidity chambers were removed and the plants were examined over a two-week period for symptom development, which was scored using the standard area diagrams for *Septoria* leaf scorch (James 1971).

3.107 The effects of humidity and temperature on spore germination and appressoria formation

Plants and spore suspensions were prepared as above. The sixth leaf was removed from five-week old wheat plants. Cultures and spore suspensions were prepared as above using two different isolates from the 1996/97 growing season. The

leaves were cut into 1 cm segments. Glass Petri dishes were lined with Whatman No 4 (9 cm diameter) qualitative filter paper which was soaked in sterile kinetin solution (0.02g/l) (to delay senescence of the wheat leaf pieces).

'Humidity' solutions were added to small glass petri dishes (average: 4 cm diameter). These solutions were made up three weeks prior to use and left to equilibrate at room temperature, with more solids being added when all solids had gone into solution. The humidities used were 55-60% RH and 98-100% RH at temperatures 10, 15, 20, 25^{0}C in a gradient temperature incubator. At 10^{0}C and 15^{0}C at 58% RH the solution used was a 1:1 mixture of ammonium nitrate and sodium nitrate (NH_4NO_3 + $NaNO_3$). To achieve 98% RH at 10^{0}C and 15^{0}C potassium dihydrogen phosphate (KH_2PO_4) was used. At 20 and 25^{0}C the solutions used were glucose ($C_6H_{12}O_6$) at 55 %RH and potassium dichromate ($K_2Cr_20_7$) at 98%RH. The small Petri dishes were placed inside the larger ones and 2.5 ml of the required humidity solution was added. The humidity solutions used were refreshed at each sample as to avoid stratification problems (Harrison *et al.* 1994).

Two isolates were used (Appendix 6), and each treatment had three replicates. The leaves were placed on kinetin-soaked filter paper. The adaxial surface of each leaf was inoculated with 20µl of spore suspension and the control leaves were inoculated using the same procedure however, 20µl of sterile deionised water with 'Tween 20' was used. The Petri dish lids were then replaced and the Petri dishes were carefully placed inside a Glad™ Snaplock bag to maintain the desired humidity level in an airtight surrounding. The Petri dishes were then placed in the incubator.

Five, 10, 25, 30, 37 and 50 h after inoculation, two leaf pieces were removed from each treatment and were subjected to leaf clearing and staining as described previously. The leaf pieces were then stored in glass Petri dishes with Whatman no. 4 (11cm diameter) filter paper soaked in glycerol. The dishes were sealed to prevent the drying out of the leaf pieces. The leaf pieces were mounted in 50% acetic acid and 50% glycerol for examination under an Olympus BH2 microscope. Percentage germination

of conidia and formation of appressoria was recorded. Results were analysed using a repeated measures ANOVA and the experiment was of a split plot design.

3.108 Fixation of material for Transmission Electron microscopy

Three extra leaf pieces per treatment from the above experiment were removed 25, 37, 50, 58, 63 and 96 h after inoculation. Sections (1mm x 1mm) of leaf tissue within the area where the drop of spore suspension was placed were excised using a scalpel under a stereomicroscope and fixed in a 5% solution of biological grade glutaraldehyde in phosphate buffer (0.025M, pH 7.2 buffer). Ten sections were fixed per sample and the vials containing the leaf material were placed under vacuum (20 mm Hg) overnight in a refrigerator. The vials were removed from the vacuum and the fixative removed. The samples were washed three times in phosphate buffer.

A 2% (w/v) solution of osmium tetroxide in distilled water was added to the vials, which were capped and left for 2h at room temperature. Subsequently the osmium tetroxide was removed with a Pasteur pipette and the samples were then subjected to a graded acetone dehydration series. After five washes in 100% acetone, the acetone was removed and approximately 2:1 mixture of 100% acetone and Spurr's resin was added to each vial. The vials were recapped and rotated at room temperature for one hour. The resin was then removed and replaced with 2:1 mixture of resin and acetone. The vials were recapped and rotated at room temperature overnight. The resin was then removed and replaced with fresh resin and rotated for 12 h at room temperature. Plastic planchettes were filled with Spurr's resin and the sections of leaf material were added. The planchettes were placed in an oven at 70^0C for 12 h.

3.109 Sectioning for Transmission Electron microscopy

Leaf material was sectioned using a LKB ultramicrotome with a diamond knife. Sections were placed on coated or uncoated 100 mesh gold grids. The sections were post-stained on the grids using ethanolic uranyl acetate for 10 min and lead citrate for 2 min. The sections were then examined under a Joel Electron Microscope - 1200 EX operating at 80KV.

3.110 Sectioning for light microscopy

Optical sections (2.5μm)of the embedded leaf material were cut using a LKB microtome and stained using Methylene blue and azure blue. These sections were examined under a compound microscope (Olympus BH2).

3.20 Results

3.201 Effect of kinetin on the radial growth of *Didymella* isolates in cultures and on the germination of conidia of *Didymella*

Kinetin had no significant effect on either the radial growth of *Didymella* in culture or on the germination of conidia.

3.202 The use of detached leaves versus attached leaves

There was no statistically significant difference between the germination of conidia and the production of appressoria on attached versus detached leaves.

3.203 The effect of different sources of inoculum on the development of scorch symptoms

Leaf scorch developed on plants that were inoculated with a disc of mycelia but not on those that were inoculated with a disc of agar. The leaf scorch lesion was directly below where the disc was resting and was two mm in diameter one week after inoculation (Plate 3.10). The discs had to be removed manually after three days as they dried to the leaves and the scorch symptoms could not be observed. Plants inoculated with spore suspensions showed no leaf scorch symptoms after the same period of incubation at high relative humidity and 20^0C. The leaves inoculated with mycelia discs showed a Safarin red colouration of the plant nuclei within the scorch lesion, and the lesion itself.

3.204 Influence of sticker solutions on the processes of infection

Conidia germinated at between three and five h after inoculation regardless of sticker solution used. Appressoria formed at between 24 and 29 h after inoculation. Three weeks after inoculation, leaf one of all inoculated plants showed less than one percent (leaf area) leaf scorching; no symptoms were noted on the control plants. No further scorch symptoms developed on any inoculated or control leaf. *Didymella* was reisolated from surface sterilised leaf tissue of all inoculated plants up to three weeks after inoculation. The amounts isolated from the leaves inoculated with different sticker solutions were not statistically significant, but more *Didymella* was isolated from leaves that had been inoculated with agar as a sticker solution. Inoculated plants were observed to senesce at a similar rate to un-inoculated plants

3.205 Influence of temperature and humidity on the germination of *Didymella* conidia on detached leaves

3.2051 Time 1 – 5 hours (see Figure 3.1)

Conidia had started germinating five hours after inoculation at 10^0C and 65% and 10^0C and 98% RH, whilst over 80% of conidia had germinated at 65% RH. Fewer conidia had germinated at 98% RH than at 65%RH. Large variation occurred within numbers of conidia germinating at 15^0C and 98% RH and 20^0C and 65% RH. Temperature was significant at five hours after inoculation. The germination rates at 20^0C and 25^0C were significantly higher than those at 10^0C, and the rate at 25^0C was significantly higher than that at 15^0C.

3.2052 Time 2 – 10 hours (see Figure 3.1)

Humidity had a significant ($p< 0.05$) effect on the percentage of conidia germinated. Germination levels were highest for those conidia incubated at 98%RH. The interaction between temperature and humidity was also significant ($p<0.05$). At 10^0C and 98% RH the numbers of conidia that germinated was significantly higher those at 65%RH. The same effect was observed at 25^0C. At 98% RH the numbers of germinated conidia was similar for both 10^0C and 25^0C.

3.2053 Time 3 - 25 hours (see Figure 3.1)

Twenty-five h after inoculation there was an interaction effect between temperature and humidity ($p < 0.05$). At 15^0C and 98% RH the level of germinated conidia was significantly lower than that at 65% RH. The lowest rate of germination was at 15^0C and 98% RH and the highest was at 20^0C and 98%RH.

3.2054 Time 4 – 30 hours (see Figure 3.1)

At 30 h after inoculation the interaction between temperature and humidity was significant ($p<0.05$). Conidial germination was higher ($p<0.05$) at 15^0C and 98% RH than at 65%RH. Germination is highest at 65%RH and 20^0C. The levels at the other temperatures and humidities apart from 15^0C were relatively similar. Over the course of the experiment humidity was the only significant effect. Temperature did not appear to play a major role in germination of conidia and the effect of both relative humidities used varied between sampling times.

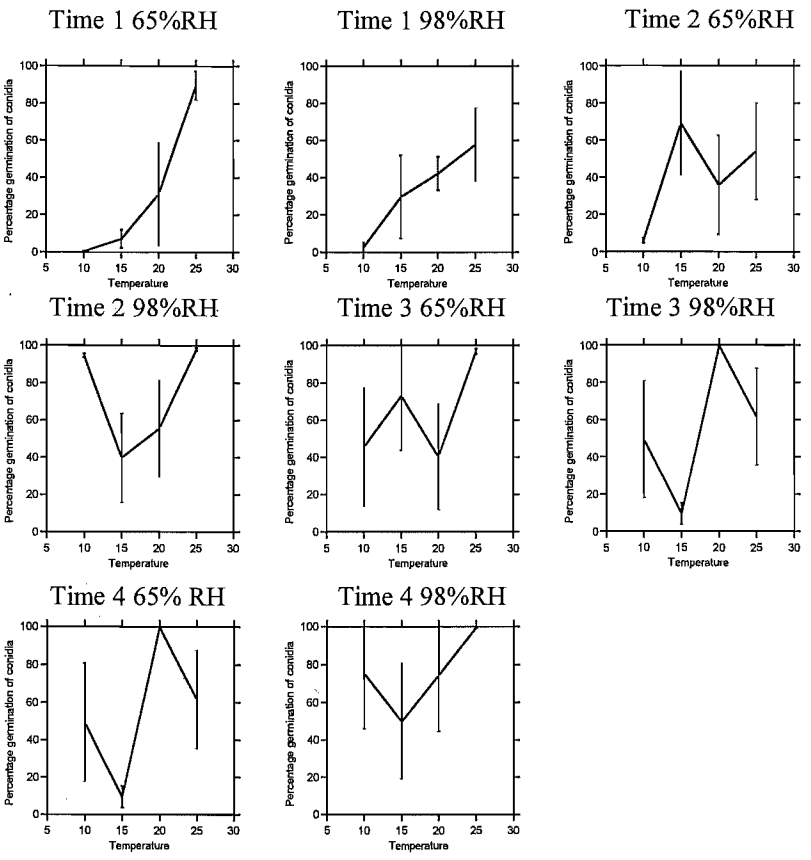

Figure 3.1 The effect of temperature and humidity on the germination of conidia on detached leaves at sampling times 1-4 (mean ± 1 standard error). Time 1 is 5 hours, time 2 is 10 hours, time 3 is 25 hours, and time 4 is 30 hours after inoculation of wheat plants cv. Monad with *Didymella* spp.

3.206 Influence of temperature and humidity on the production of appressoria on detached leaves

3.2061Time 5 – 37 hours (see Figure 3.2)

Very few appressoria were produced until time five, so sampling times 1-4 were not included in the repeated measures ANOVA. At 37 h after inoculation temperature ($p< 0.05$), humidity ($p< 0.005$) and the interaction effects between temperature and isolate ($p<0.05$) and temperature*humidity* isolate ($p<0.05$) were significant. The number of appressoria produced was higher at 20^0C than at 10^0C and more appressoria were produced at 98% RH than 65%RH. Large variation within the sample occurred at

25^{0}C and 98% RH. The interaction between temperature and humidity increased the numbers of conidia germinated at 20^{0}C and 98% RH than the level at 65%RH and 20^{0}C. More appressoria were produced at 25^{0}C and 65%RH than at 10^{0}C and 98% RH

3.2062 Time 6 – 50 hours (see Figure 3.2)

Fifty hours after inoculation temperature ($p<0.01$) had a significant effect on the numbers of appressoria produced. The number produced at 10^{0}C and 15^{0}C were significantly lower than the number produced at 25^{0}C.

3.2063 Time 7 – 58 hours (See Figure 3.2)

At 58 h after inoculation humidity had a significant effect on the numbers of appressoria produced. More appressoria were produced at 65%RH than at 98%RH. Fifty eight h after inoculation the number of appressoria produced at 10^{0}C had increased to the levels present at the other temperatures.

3.2064 Time 8 – 63 hours (See Figure 3.2)

No treatment was significant at 63 h after inoculation. The formation of appressoria was highest at 25^{0}C but there was very little difference between the other temperatures and humidities. Temperature had a significant effect on the production of appressoria over the period of the experiment.

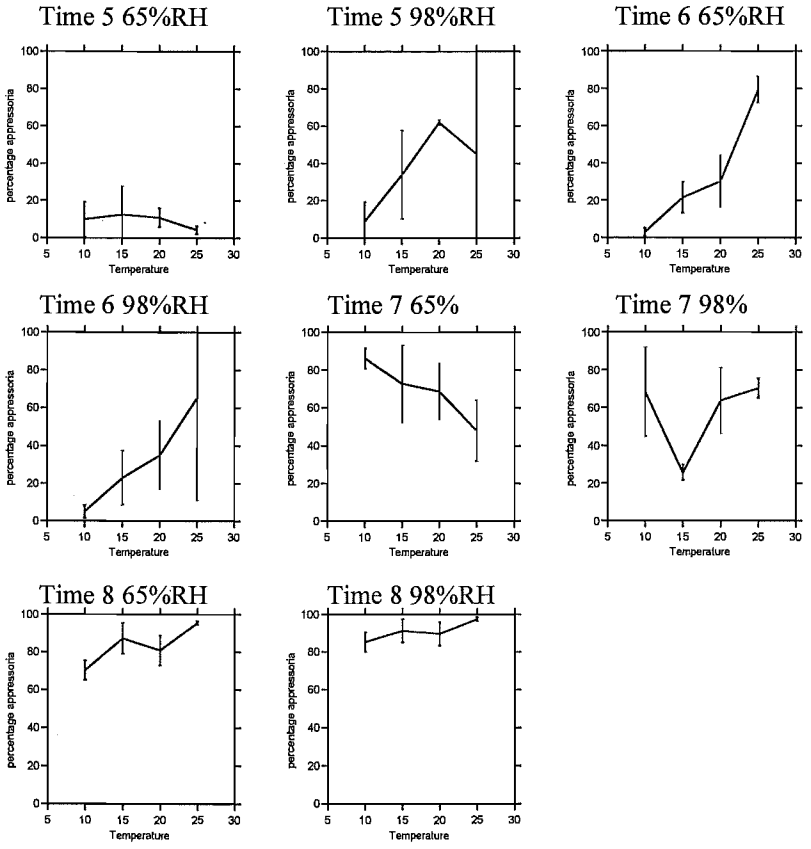

Figure 3.2 The effects of temperature and humidity on the production of appressoria at sampling times 5-8 (mean ± 1 standard error).). Time 5 is 37 hours, time 6 is 50 hours, time 7 is 58 hours, and time 8 is 63 hours after inoculation of wheat plants cv. Monad with *Didymella* spp.

There was no specific orientation of germ tubes on the leaf surface. Penetration of the leaf surface occurred between 63 and 96 h after inoculation. The mode of penetration was direct either by way of mechanical or chemical means (Plate 3.2). Invasion via stomata or stomatal subsidiary cells was never observed. Appressoria were produced randomly over the leaf surface, there were no specific positions for penetration of the leaf tissue. Conidia also produced up to three germ tubes (Plate 3.1). The length of the germ tube before the production of appressoria was variable. Blue halos were observed around appressoria (Plate 3.3).

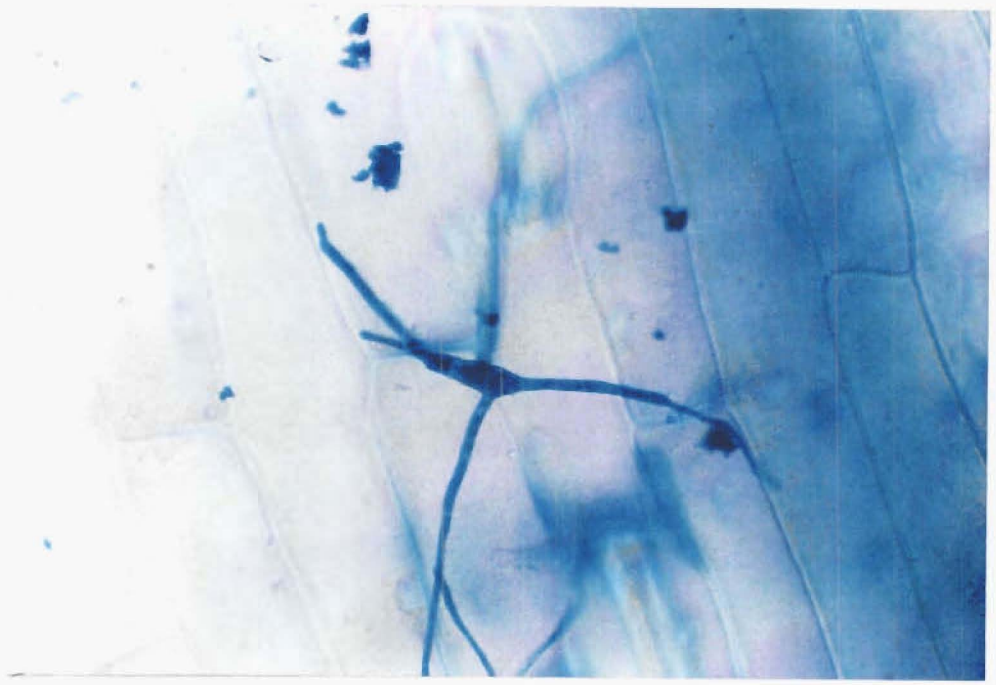

Plate 3.1 Conidium with three germ tubes.

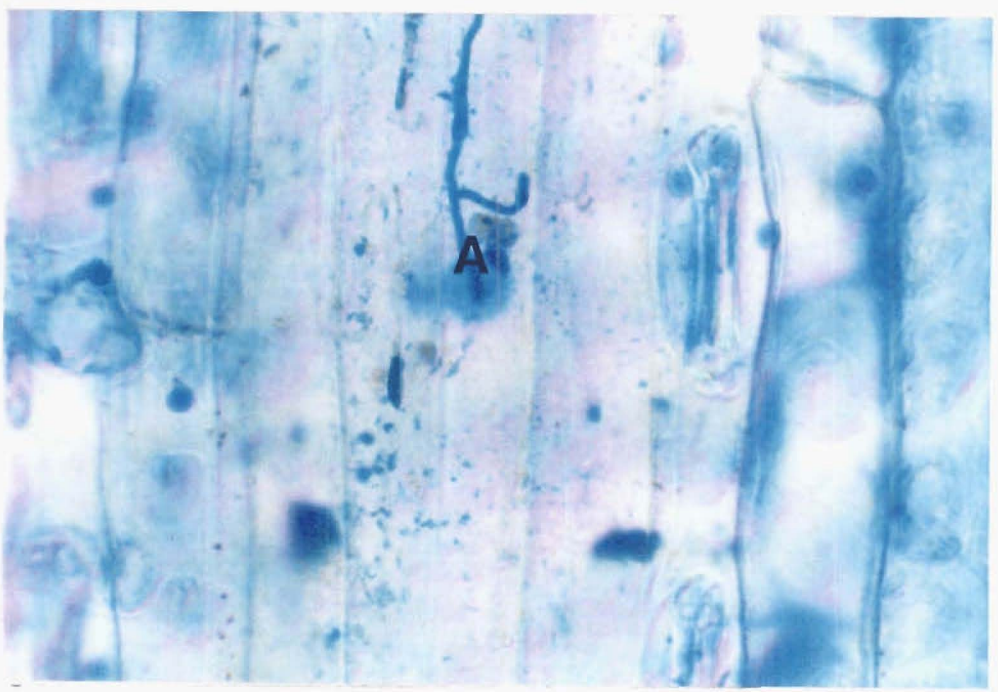

Plate 3.2 Direct penetration of the leaf tissue by the appressoria of *Didymella* spp.

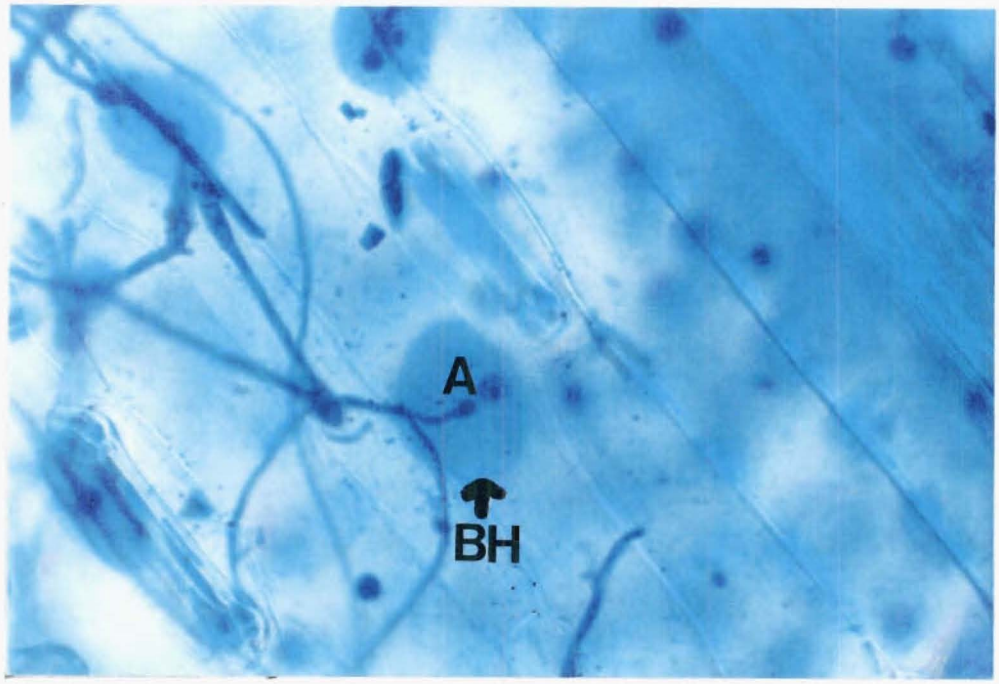

Plate 3.3 The blue halo below the appressorium.

3.207 Effect of *Didymella* species infection on the host

Conidia were easily dislodged from the leaf surface until 58h after inoculation and the processing required for TEM meant that no conidia remained on the leaf surface. No primary hyphae were found in any of the sections examined, showing that although appressoria had been produced no infection of the host had occurred. Appressoria were observed on the leaf surface after 58h. Thickening of the host cell wall was observed below the appressoria (Plate 3.9). Penetration of the host occurred between 63 and 96h after inoculation. *Didymella* penetrated directly and primary hyphae grew between the cuticle and the epidermis (Plates 3.4, 3.5, 3.7, 3.8). In most leaves examined hyphae were not observed to have ramified through the host tissue and the level of fungal invasion was the same whether the leaf tissue was incubated at 20^0C or 25^0C or the differing humidities. No visible scorch symptoms developed on the leaf tissue apart from two leaf pieces where a typical *Didymella* scorch lesion developed (Plate 3.10). The transmission electron microscope revealed that hyphae had ramified throughout leaf tissue and cell walls were broken down (cell walls were not observed to be broken down prior to infection) (Plate 3.6). Uninoculated control leaves had intact cell walls and the cytoplasm of the mesophyll cells did not appear granular. The cytoplasm of the uninfected mesophyll cells adjacent to mesophyll cells with hyphae present, appeared very granular and was no longer attached to the cell wall. Cellular disorganisation in these leaves was common as a result of infection (Plate 3.11).

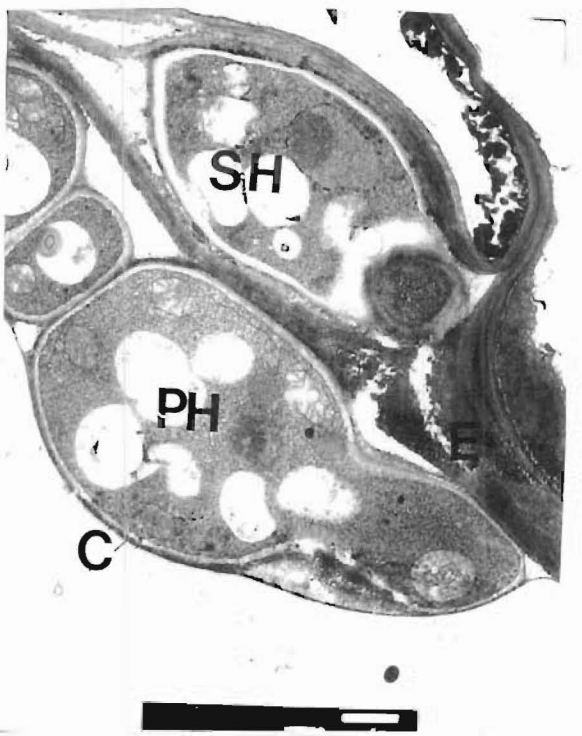

Plate 3.4 Transmission electron micrograph showing primary hyphae (PH) present just below the cuticle and secondary hyphae (SH) within the epidermal layers of wheat cv. Monad 96 h after inoculation.

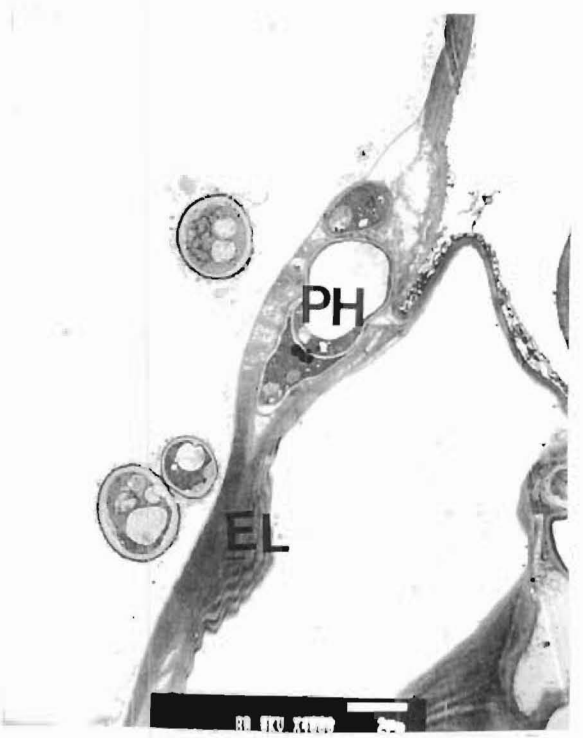

Plate 3.5 Transmission electron micrograph showing primary hyphae (PH) within the epidermal layers (EL) of wheat cv. Monad 96 hr after inoculation.

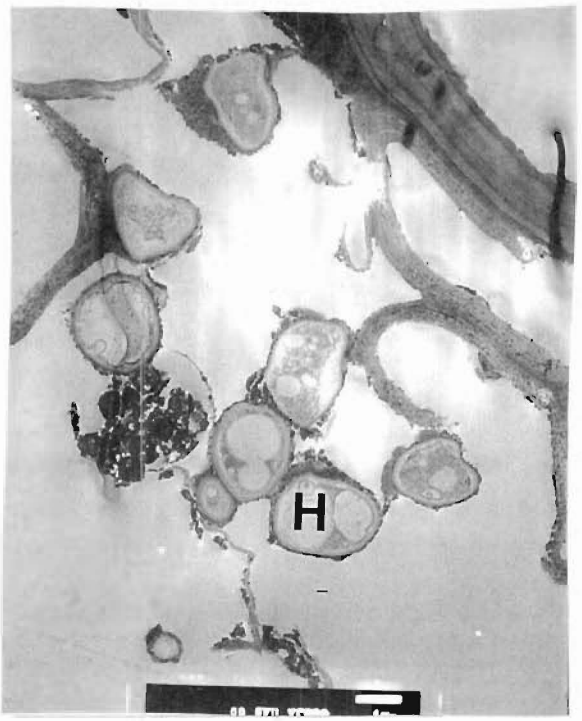

Plate 3.6 Transmission electron micrograph showing hyphae (H) present within the leaf mesophyll of wheat cv. Monad 96 h after inoculation.

Plate 3.7 Transmission electron micrograph showing primary hyphae (PH) causing infolding of the epidermis of wheat cv. Monad 96 h after inoculation.

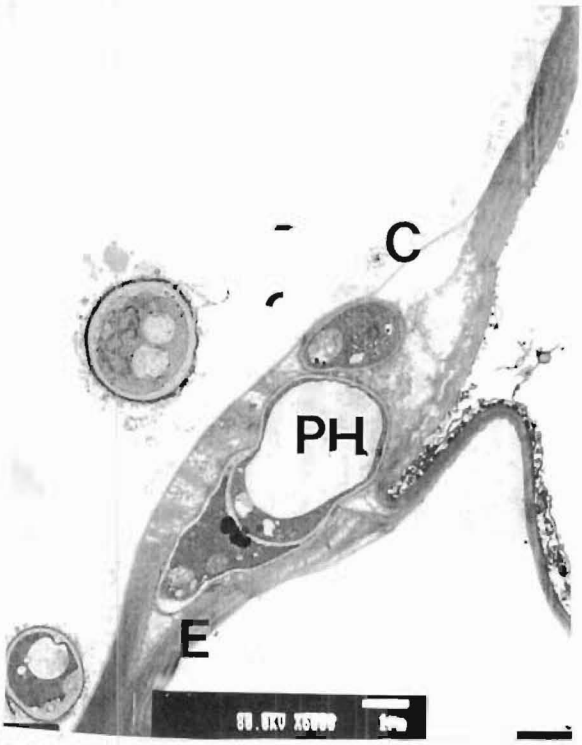

Plate 3.8 Transmission electron micrograph showing primary hyphae (PH) present between the cuticle (C) and the epidermis (E) of wheat cv. Monad 96 h after inoculation.

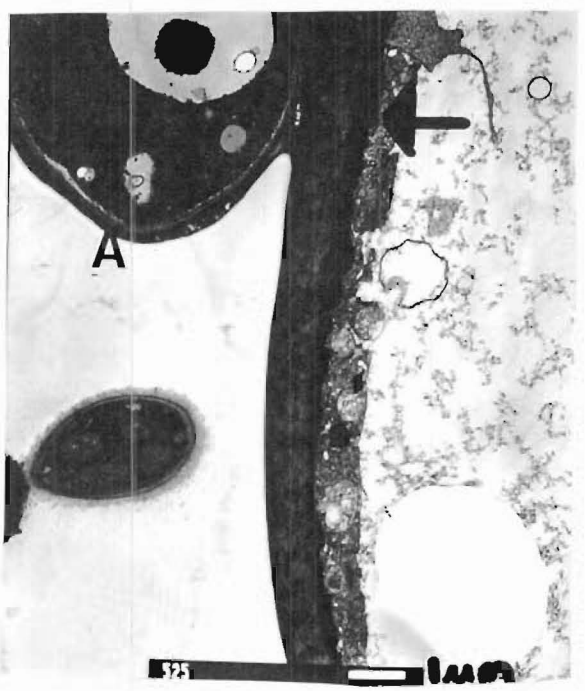

Plate 3.9 Transmission electron micrograph of wall thickening (arrow) in response to an appressorium (A) of *Didymella* spp 63 h after inoculation.

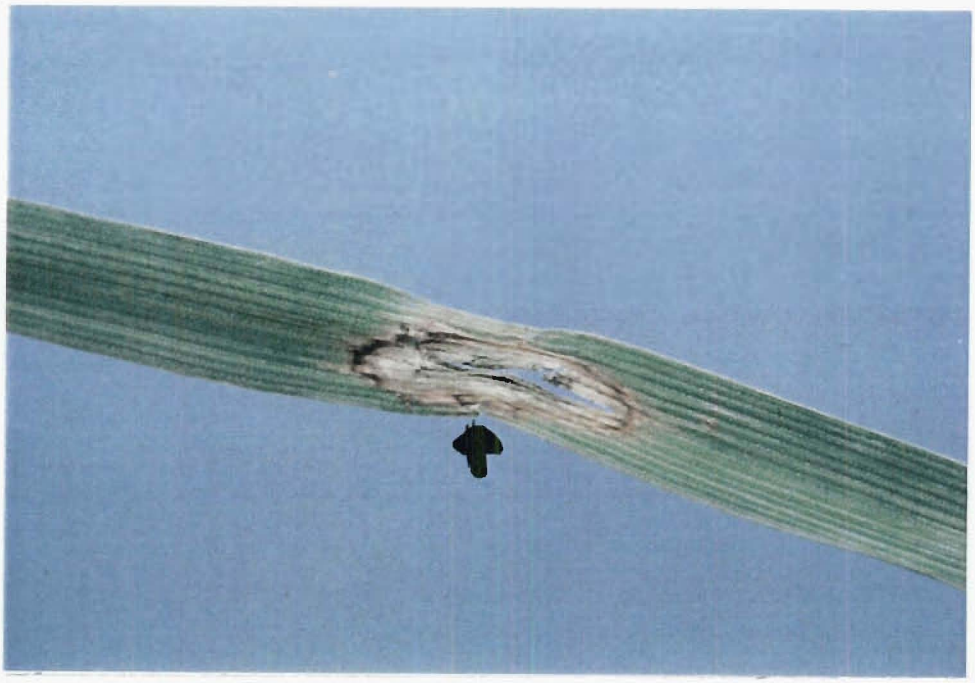

Plate 3.10 Photo-micrograph of a typical *Didymella* scorch lesion. Note the tan centre (arrow) with the dark brown border clearly delimiting the scorch lesion from the green leaf tissue.

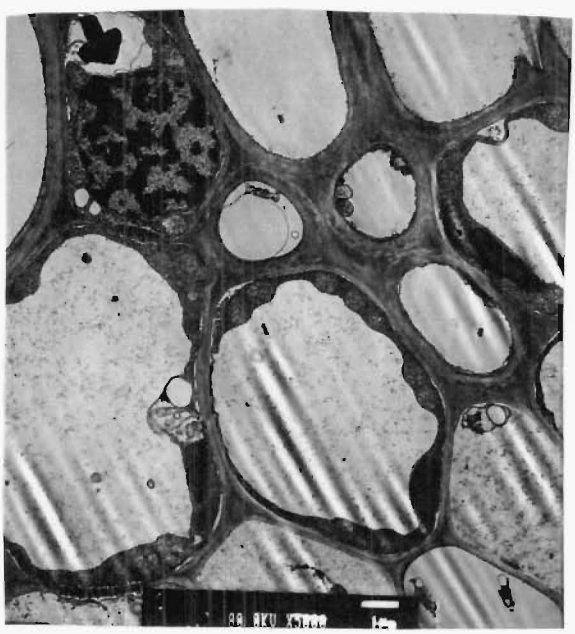

Plate 3.11 Transmission electron micrograph of the granular cytoplasm of a mesophyll cell of wheat cv. Monad leaf which showed a visible scorch symptom four days after inoculation with *Didymella* spp.

3.30 Discussion

Many factors play decisive roles not only in the initial invasion but also in the ensuing spread of diseases of plants by pathogens (Tarr 1972). Endophytic fungi are also affected by these factors as they must infect to establish an endophytic relationship.

For any pathogenic or even endophytic fungus to be successful, germination must occur rapidly (apart from in the case of resting spores) as penetration of the host must occur before conditions on the leaf surface become unfavourable. A major problem is replicating field conditions within a laboratory. Humidity in the field is constantly changing while in the laboratory it is usually relatively constant. The laboratory situation is only testing a small subset of the conditions that would influence infection in the field, and almost complete control is exercised within the laboratory, whereas in the field situation this is not possible. Hence, at best laboratory conditions can only ever approximate those present in the field. Leaf scorch symptoms are only occasionally observed on inoculated plants in the laboratory and affect on average less than 1% of the leaf area. Under field conditions leaf scorch can affect greater than 1% of the leaf area of a host plant, this difference indicates that certain abiotic and biotic conditions are potentially necessary for the formation of scorch symptoms and these are not being replicated in the laboratory.

The optimal conditions for *Didymella* spore germination is 25^0C and 98% RH, between three and five h after inoculation and germination reaches it maximum level at this time. The effects of free water were not examined in this study. At low temperatures (*i.e.* 10^0C) it took until 25 h after inoculation to reach the same level of germination as was obtained five h after germination at 25^0C. The effect of isolate was significant at five h after inoculation, but did not have any effect during the remainder of the experiment showing there were no differences between the rate of conidial germination and appressorial production in different graminicolous species of *Didymella*. The level of relative humidity would not have been at 65% initially due to the fact water was present in the droplet of spore suspension. This may have lead to

some of the inconsistencies in the numbers of spores germinated up until 10 h after inoculation

Other pathogenic fungi have specific requirements for leaf age and humidity for infection to occur (Holmes and Colhoun 1974). Different species of *Septoria* have differing humidity requirements; *S. nodorum* requires a temperature of 12^0C, plants between growth stages 6-10.5 and three h of high humidity for infection to occur, while *S. tritici* requires 20 h at this temperature and humidity. This is an indication of the differences between related species as to the conditions required for infection. *Didymella* required relative humidity approaching 100% for conidia to germinate throughout the course of the experiment whereas temperature requirements tended to be time specific.

A distinct appressorium was formed in all cases but in other *Ascochyta* spp. this is not always a prerequisite for penetration (Heath and Wood 1969, Brewer and McNeill 1953 in Roundhill *et al.* 1995). Penetration of epidermal cells occurred directly with no preferred sites, and there was no evidence of stomatal penetration or of guard cell penetration. For pathogenic fungi the preferred site for penetration often appears to be the junction between epidermal cells (Preece *et al.* 1967, Lapp and Skoropad 1978). Outward displacement of the cuticle occurs with the subsequent growth of the subcuticular hyphae within four days of inoculation. After four days, hyphae enter the mesophyll, causing the destruction of the mesophyll cells and producing the macroscopic scorch symptoms in the two leaves where symptom production was observed.

The observation that conidia and appressoria were not firmly attached to the leaf surface was unusual as most plant pathogenic fungal spores secrete material that adheres the conidia to the leaf (Paus and Raa 1973). Further investigation needs to be carried out to confirm this observation and perhaps look at the effects of natural weathering on infection of host plants by *Didymella* spp.

The response of the host to infection appears to be limited. Mace (1994 unpublished) found that the cytoplasm became granular in appearance and the cytoplasm contracted away from the cell wall, 96 h after inoculation. The current study has shown that the cytoplasm accumulates near the infection site, and localised thickening of the cell wall occurs. In two samples, the cytoplasm of the uninfected mesophyll cells became granular in appearance and secondary hyphae were observed to be present in some mesophyll cells. Fungi that enter a biotrophic phase tend to grow until the level of sugar declines within the apoplast at which point the necrotrophic phase develops (Hancock and Huisman 1981).

Subcuticular intramural growth is common among many plant pathogenic fungi, for example *Rhynchosporium secalis* (Ayres and Owen 1971, Jones and Ayres 1974, Lyngs Jørgensen *et al.* 1993) and many *Colletotrichum* spp. (Bailey *et al.* 1990, O'Connell and Bailey 1991, Pring *et al.* 1995). *Rhynchosporium secalis,* the causal agent of barley leaf-scald, causes epidermal cell collapse within 10 days after inoculation and the chloroplasts of mesophyll cells show degeneration by the 10th day after inoculation (Jones and Ayres, 1974). The length of the biotrophic phase differs between hemibiotrophs, some having a biotrophic phase of a few days, for example, *R. secalis* (Jones and Ayres 1974) on barley, whereas, *V. inaequalis* on apple is several weeks in duration (Heitefuss and Williams 1976).

The staining method used allowed the visualisation of the changes taking place in the host cell. A halo developed directly over the point of penetration due to an accumulation of silicon and manganese and protein (Kunoh *et al.* 1975 in Wolf and Fric 1981). This allows visualisation of penetration showing that in some instances it did occur earlier than between 63 and 98 h.

Inoculum applied as mycelial blocks increased the expression of scorch symptom occurring on the leaf tissue directly underneath the mycelial block. The mycelial blocks were left on the leaf until they had completely dried as removing them before this time caused damage to the leaf tissue. This gave the mycelia longer periods of moisture for

infection to occur. The blocks were also difficult to dislodge, unlike spores that are relatively easy to dislodge even with the use of sticker solutions. No un-inoculated (*i.e.* leaves inoculated with agar blocks only) leaves showed symptoms and the rate of senescence of the inoculated leaves was no faster than the control leaves. Both the spore suspension and mycelial block inoculated leaves produced a red colouration on the leaves. The scorch lesion and the plant cell nuclei in and around the scorch lesion appeared to be stained a Safarin red. *Didymella* in culture produces a brick red pigment on PDA and it is thought that it was the production of this pigment that caused the red colouration of the leaf tissue, however nothing is known of this pigment.

There was no effect of leaf age at the time of inoculation on the production of symptoms, the rate of senescence, nor on the rate of conidial germination or appressorial production. The production of scorch symptoms was not consistent even when the infection conditions were identical between trials. There was considerable variation in symptom expression, if symptoms were produced at all they could be produced within a day or within a month after inoculation.

The indication from this study is that *Didymella* is endophytic in nature as spore germination at less favourable humidities and temperatures is not delayed for more than 10 h, but if the conditions (whether they are biotic or abiotic in nature) become favourable scorch symptoms can be produced. These symptoms affect on average less than one percent of the leaf area and do not increase the rate of senescence. It is present in a subcuticular intramural location within the epidermis until the leaf senesces. At this point *Didymella* is triggered to leave this location and ramify through the leaf tissue at which point sporulation appears to be stimulated. The release of nutrients from the plant cells may stimulate *Didymella* sporulation. The evidence for this is the sporulation of *Didymella* within a paraquat spray drift lesion (see Chapter 4 for further discussion). The paraquat acts to cause cell death and hence the release of cell nutrients which may trigger the growth of *Didymella* from its subcuticular intramural location. Chapter four examined the effects of paraquat on leaf laminar infected with *Didymella* spp in the laboratory. No scorch symptoms were obtained in the laboratory. It seems likely that the

conditions in the field were not replicated sufficiently thus no sporulation of *Didymella* was observed. It appears from the research done for the current study that *Didymella* has a requirement for a set combination of abiotic and possibly biotic conditions for sporulation to occur but at the present time this combination is unknown.

In summary, the optimal conditions for the development of leaf scorch were not established. The optimal conditions required for conidial germination were found to be at 25^0C and 98% RH. For the production of appressoria the conditions required were 20^0C and 98%RH. The production of symptoms was not consistent and leaf scorch only affected at most one percent of the leaf area. *Didymella* was always recultured from inoculated leaf material. *Didymella* did not increase the rate of senescence of inoculated plants. In conclusion it appears that *Didymella* is either an endophyte or a weak pathogen which remains in a subcuticular intramural location until leaf senescence starts to occur at which point it starts to ramify through the host tissue.

4.0 IN-VITRO INVESTIGATIONS OF *DIDYMELLA* SPECIES

4.01 Introduction

The classification of any fungus depends on a detailed knowledge of its life cycle (Simmons 1952). Such observations are often difficult to make, due to problems in obtaining significant data, or inaccurate observations due to technical difficulties. There are two main factors that allow for accurate descriptions to be made. These are; to have an understanding of the pattern of variation in natural populations, and knowledge of the correlation between the anamorph and the teleomorph states. There is often difficulty in obtaining knowledge about these factors as cytological and morphological work can be hampered by the difficulty of obtaining one or both states in culture, or technical staining and interpretation problems. Often the anamorph state has tenuous links with the teleomorph state and once obtained in a pure state may not accurately reflect the variation in field isolates.

4.02 The effects of nutrients on sporulation of fungi

Little is known about what conditions filamentous fungi require in order to promote teleomorph development, however, it is known that that they require an exogenous supply of vitamins (Tremaine and Miller 1954). The types and quantities of available nutrients and growth factors often affect the production of fruiting bodies on synthetic media by fungi (Campbell 1958). As thiamine concentration was reduced perithecial formation was inhibited in *Ceratostomella* (=*Ceratocystis*) *fimbriata* (Ellis and Halst) Elliot (Barnett and Lilly 1947b). Hawker (1939 in Ross 1961) found that *Melanospora destruens* required biotin for growth, but thiamine was necessary for perithecial development.

Calcium and varying nitrogen levels are required for the formation of perithecia of several species of *Chaetomium* (Basu 1951 in Campbell 1958). Different forms of nitrogen can also affect mycelial growth and perithecial formation. *Ceratocystis variospora* (Davids) Moreau sporulated best on media containing mixtures of asparagine and calcium nitrate in a 1:3 ratio that gave a total nitrogen content of 0.636g/l (Jailloux 1992). Mycelial growth was best on asparagine alone (Lactoste 1965

in Jailloux 1992, Fayret 1975 in Jailloux 1992, Assemat and Fayret 1987 in Jailloux 1992). Low nutrient substrates are often successful in inducing the formation of teleomorphs. The formation of the teleomorph of *A. avenae* (Petrak) Sprague and Johnstone, was induced on carrot juice agar (Obst 1983).

4.03 The effects of different inoculum sources

It was found by Lilly and Barnett (1951) that the sporulation of some fungi is more abundant when a spore suspension is used to flood the agar plate surface than when inoculum is placed in the centre of the plate. Three reasons have been suggested to explain this observation. The first is that nutrients are exhausted more rapidly (Timnick *et al.* 1952 in McOnie 1964), the second that growth factors within the spore matrix encourage sporulation (Mathur 1951 in McOnie 1964), and third that sporulation inducing factors may be carried over within the spores (Lilly and Barnett 1964 in McOnie 1964).

4.04 Effects of temperature, light and humidity on sporulation

Temperature, light, and humidity often affect sporulation of fungi. Conidia of *Guignardia bidwellii* were produced on oatmeal agar by the manipulation of light and temperature (Jailloux 1992). Sweet (1942 in Lilly and Barnett 1951) found that relative humidity had some effect on the production of perithecia and conidia of two species of *Magnusia* but the response was varied, whilst Goldring (1936 in Lilly and Barnett 1951) found that there was little or no effect of relative humidity on the production of sporangia and sporangiola of *Blakeslea trispora* strains. A large range of species of fungi can be induced to sporulate the anamorph state using exposure to near UV light (Leach 1962).

4.05 Use of herbicides in the sporulation of fungi

Herbicides, for example paraquat, have been used by a few researchers to detect latent infections of fruit or in plant tissue (Cerkauskas and Sinclair 1980, Biggs 1995). The sporulation of some fungi present in plant tissues is affected by paraquat most likely due to the altered relationships with saprophytic fungi which may be reduced after paraquat application. On detached leaf segments the mycelial growth and the

sporulation of *Septoria nodorum* is inhibited by paraquat (Cerkauskas and Sinclair 1980).

4.06 Use of plant parts to encourage sporulation

Wheat plant parts such as the straw, leaves and the grains have been used by many researchers to encourage fungi to sporulate (Kirby 1925 in Holden and Hornby 1981, Bussman 1936 in Holden and Hornby 1981). The development of perithecia and ascospores may be rapid (within 3-4 weeks on agar) (Weste and Thrower 1963, Willets 1961 in Holden and Hornby 1981), but more often their development on plant substrates takes longer than on agar (Padwick 1939 in Holden and Hornby 1981, Nilsson 1969 in Holden and Hornby 1981, Willets 1961 in Holden and Hornby 1981).

4.07 The effects of oxygen on sporulation

The effect of oxygen on cultures is not very well studied, however it is thought it may play a role. Most fungal cultures are kept closed with little or no ventilation and often the humidity is high in the plates. This may in fact adversely affect the sporulation of the fungus, due to the accumulation of gases from its metabolism (Barnett and Lilly 1955).

4.08 Aims

The aims of this chapter are to:

1/Attempt to consistently produce the teleomorph state of *Didymella* in culture.

2/ establish the optimum temperature and pH for growth of cultures

4.10 Materials and Methods

Initially, a range of different media were trialed to see if the teleomorph state could be produced in culture. Isolates from long term storage at -80^{0}C were grown on PDA and then, using a no. 2 cork borer, regions of actively growing mycelium from four day old cultures were plated on to the different media. Cultures were then incubated at 18^{0}C under 12 h darkness and 12 h light for seven days before cultures were removed and scraped with a sterile microscope slide and placed under a Philips black light at 18-20^{0}C. Twenty isolates were trialed on each of the different media and

for each of the 20 isolates there were two replicates. All media were sterilised at 15lbs/in^2 (121^{0}C) for 15 minutes. Fifteen ml of media was placed in each petri dish. Where heat sensitive compounds, for example vitamins, were required, these compounds were filter sterilised and added after the medium was autoclaved. See appendix 7 for the isolates used to investigate whether or not the teleomorph could be produced in vitro.

4.101 Media used (Tuite 1969 and Dhingra and Sinclair 1985)

Alphacel medium

Alphacel	20g
$MgS0_47H_20$	1g
KH_2PO_4	1.5g
$NaNO_3$	1g
coconut milk	50ml
agar	12g
distilled water	1l
adjust pH to 5.6	

Other variations trialed were the addition of 10g of tomato paste or 10g of oatmeal or autoclaved wheat straw to 1l of the medium.

Asthana and Hawker medium A

glucose	5.0g
KNO_3	3.5g
KH_2PO_4	1.75g
$MgSO_4$	0.75g
agar	15g
dist water	1l

Barnett Maltose Casamino medium

maltose	5g

Difco casamino acids	1.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
Zn	0.2 as sulfates
Fe	0.2
Mn	0.1
Biotin	5µg/ 0.5g yeast extract
water	1l
agar	20g

adjust to pH 6

Adaptation on the above medium: Difco casamino acids 2g/l replacing asparagine as Nitrogen source and amount of glucose was increased from 2 to 3g.

Browns medium

glucose	2g
K_2HPO_4	1.25g
agar	20g
asparagine	2g
$MgSO_4$	0.75g
water	1l

Carnation leaf agar

Autoclaved carnation leaves were added to molten PDA and water agar plates

Czapek-Dox

$NaNO_3$	2g
KH_2PO_4	1g
$MgSO_4.7H_2O$	0.5g
KCl	0.5g
$FeSO_4$	0.01g
Sucrose	30g
Agar	20g

water 1l

Different nitrogen sources instead of sodium nitrate:

nine amino acids giving a final concentration of 0.42 g/l Nitrogen

b- alanine

DL serine

DL cytseine

DL tryptophan

L-tyrosine

L-leucine

L-Histidine

glycine

DL valine

Different sources of carbon were also trialed these being fructose, glucose, maltose or sucrose

Czapek-dox V-8® juice agar

modified CD	45.4g
$CaCO_3$	3g
water	800ml
V8® juice	200ml
agar	10g

Difco Cornmeal agar

Difco Lima bean agar

Gibco PDA

Lactose Casein hydrolysate medium

lactose	37.5g
casein hydrolysate	3.0g
KH_2PO_4	1.0g

$MgSO_4$	0.5g
microelements	2ml
agar	15g
water	1l

adjust to pH 6

microelement solution

$Fe(NO_3)_3.9H_2O$	723.5mg
$ZnSO_4.7H_2O$	439.8mg
$MnSO_4.4H_2O$	203.0mg

dissolve one at a time in 1l water add sulfuric acid to clear the solution

Leonian

peptone	0.625g
maltose	6.25g
malt extract	6.25g
KH_2PO_4	1.25g
$MgSO_4.7H_20$	0.625g
agar	20g
water 1l	

Lilly and Barnett agar 0.1% yeast extract substitute for thiamine and biotin

Lilly and Barnett 1% glucose and 0.2% asparagine

2.0% glucose and 0.4% asparagine

Malt agar Leonian version

malt extract	3g
yeast extract	2g
KH_2PO_4	0.5g
$MgSO_4.7H_2O$	0.5g
agar	20g
water	1l

Malt extract broth (Difco) 20 ml of 1% w/v malt extract broth and wheat straw

Merck PDA.

Milk PDA 5 g of Anchor powdered milk in PDA (39g/l)

Mineral peptone agar

KH_2PO_4	1.25g
$MgSO_4.7H_2O$	0.625g
peptone	0.625g
agar	20g
distilled water	1l

***Neurospora* complete medium**

glucose	5.0g
sucrose	5.0g
hydrolysed casein	5.0ml
yeast extract	2.5g
spray dried malt syrup	5.0g
vitamin solution	10ml
agar	15g

vitamin solution	**mg/l**
thiamine	100
riboflavin	50
pyridoxine	50
pantothenic acid	200
p- aminobenzoic acid	50
nicotinamide	200
choline	200
inositol	400

alkali hydrolysed yeast nucleic acid	500
folic acid	4 µl

***Neurospora* minimal medium**

ammonium tartrate		5.0g
NH4NO3		1.0g
KH_2PO_4		1.0g
$MgSO_4.7H_20$		1.0g
NaCl		0.1g
$CaCl_2$		0.1g
sucrose	15g	
biotin		5×10^{-6}g
Bo		0.01mg
Cu		0.1mg
Fe		0.2mg
Mn		0.02mg
Mo		0.02mg
Zn		2.0mg

pH 5.6

***Neurospora* sex synthetic** trace elements as above

KNO_3		1g
KH_2PO_4		1g
$MgSO_4$		0.5g
$CaCl_2$		0.1
NaCl		0.1
biotin		5µg/l
trace elements		1ml
sucrose	20g	
agar		15g
water		1l

adjust to pH 6.5 before autoclaving

Oatmeal agar (Difco)

Potato carrot agar (Difco)

Potato marmite agar PMA

dextrose	20g
potatoes	250g
marmite	1.0g
agar	20g

$^1/_{10}$ **PDA** 3.9g PDA, 20g agar, glucose 20g/l water

PDA and malt extract Gibco PDA amended with Difco malt extract

PDA yeast extract $^1/_4$ PDA + 0.2% w/v Difco yeast extract

Prune agar (Difco)

Ross' agar

KH_2PO_4	1.5g
$MgSO_4.7H_2O$	0.5g
glucose	5.0g
Tween 80	1.0g

trace elements (µg/l)

boron	5
manganese	10
copper	20
zinc	90
$iron^{2+}$	100

cobalt	100

vitamins µg/l

thiamine	250
inositol	1000
pantothenic acid	500
nicotinic acid	500
pyridoxine	125

agar	17g
distilled water	1l

Sach's agar

$CaNO_3$	1.0g
K_2HPO_4	0.25g
$MgSO_4$	0.25g
$FeCl_3$	trace
$CaCO_3$	4.0g
agar	20g
water	1l

***Septoria nodorum* sporulation agar**

carbon source	20g
$MgSO_4.\ 7H_2O$	0.5g
Zn^{++}	0.2mg
agar	20g
nitrogen source	0.425g N
Fe^{+++}	0.2mg
Mn^{++}	0.1mg
water	1l

Alternative carbon sources: galactose or maltose, glucose + sucrose (1:1) or sucrose alone.

Nitrogen sources trialed: glycine, urea or asparagine.

Trace element solution for 100ml

$Fe_2(SO_4)_3$	36mg
$MnSO_4$	13.75mg
$ZnSO_4$	24.8

Add 2ml to 1l medium

Trione mixed cereal grains

mixed cereal grains	50g
sucrose	15g
agar	15g
thiamine HCl	5mg

This recipe was altered to include wheat straw instead of cereal grains

V_8 juice agar

V_8 vegetable juice	200ml
Agar	20g
calcium carbonate	4g

pH adjusted to 7.3 with KOH

Water agar 1l of water and 20g of agar

Water agar + $NaN0_3$ and $MgSO_4$

Wheat leaf decotion agar

Wheat leaves were simmered in 100 ml of water and then extract was made up to 1l and 15g of agar was added

½ strength version also used

Wheat straw agar

$NaNO_3$	3g
$MgSO_4$	1g
agar	20g
water	1l

Wheat straw and malt extract broth

100ml of malt extract broth and 6 pieces of wheat straw per conical flask.

Yeast extract agar (Difco)

Seven days after inoculating the agar media above, half the cultures were scraped with a sterile microscope slide. The level of sporulation of the anamorph state was recorded 14 days after the isolates were plated. Sporulation was scored as sparse (less than 25% of the culture covered with pycnidia), medium (between 25 and 65% of the culture covered with pycnidia) and abundant (more than 65% of the culture covered with pycnidia).

4.102 Light conditions

Isolates plated on the above-described media were exposed to:

1/ a Philips black light (UV 350 nm) plus a white fluorescent light with alternating 12 h light and 12 h dark;

2/ direct sunlight (cultures were placed outside in the summer);

3/ sunlight through glass;

4/ plates were wrapped in coloured cellophane (blue, red, or green) and placed under near - UV light or sunlight thorough glass.

5/ cultures were incubated in the dark, or in the dark for 10 days and transferred to light conditions or incubated in the light for 10 days and then incubated in the dark.

4.103 Carbon and Nitrogen sources

Using *Septoria nodorum* sporulation agar and Czapek Dox medium, different sources of carbon and nitrogen were tested to see what effects these would have on the sporulation of *Didymella.* The carbon sources used were galactose, maltose, glucose + sucrose, sucrose, fructose and citric acid. The different nitrogen sources tested were glycine, urea, asparagine, asparatic acid and sodium nitrate. Lilly and Barnett medium was also used with differing amounts of glucose and asparagine. Glucose at 1% and 0.2% asparagine was trialed as was 2.0% glucose and 0.4% asparagine. Casamino acids (2g/l) replaced asparagine as the nitrogen source and the glucose was increased from 2 to 3g. in another variation on the basal medium (Barnett and Lilly 1950). Further to these trials the Barnett and Lilly medium with glucose and thiamine at different concentrations (Table 4.1) was investigated as to the effect on the production of perithecia in culture (Barnett and Lilly 1950).

Table 4.1 The range of different glucose and thiamine concentrations in Barnett and Lilly medium used to investigate the effects of different concentrations of glucose and thiamine on the growth and sporulation of *Didymella* in culture.

glucose (g/l)	Thiamine (µg/l)
25	1.0
25	1.5
25	25
2	25

Half of the isolates plated on the different media had the lids removed from the Petri dishes in a laminar flow cabinet to allow aeration of the plates for five min per day for 14 days.

4.104 Ranges of pH trialed in buffered PDA

The growth rate of *Ascochyta* at different pH ranges was investigated. Table 4.2 shows the different pH used in buffered PDA (appendix 1). Ten isolates were plated on the media with three replicates per treatment. The cultures were then incubated at the 20^0C temperature and the radial growth of the cultures was measured 24, 48 and 72 h after plating.

Table 4.2 The range of pH in buffered PDA used to investigate the effects of pH on the growth of *Didymella* in culture.

4.8-5.1

5.8-6.1

6.2-6.6

7.2-7.5

8.5-8.6

4.105 Mating isolates

A range of 10 isolates was selected at random from the 95/96 and 96/97 collections of isolates (Appendix 7). These isolates were plated in all combinations possible on PDA initially and then on all the media used previously. Two mycelial plugs from two different isolates were plated on to PDA two cm apart and then the cultures were incubated at 18-20^{0}C and examined every week for pycnidial and perithecial production. Mechanical injury of half the cultures was carried out by scraping half of the culture with a sterile microscope slide. Fourteen days after inoculation plates were placed under near - UV light and examination of the plates continued weekly.

4.106 Different inoculum sources and ages of inoculum

Age of inoculum has been observed to have an effect on the sporulation of fungi so the following ranges of culture ages were used to inoculate all the above media using the standard technique described above. The age of the inoculum was as follows 7, 14, 21, 28 days after initial plating. Spore suspensions were prepared by adding sterile distilled water to the plate and scraping with a sterile microscope slide 10, 14 day old sporulating cultures that had been grown on PDA. The resulting suspension was filtered through sterile muslin and 20µl was pipetted on to the different media and was distributed evenly over the plate surface using a glass spreader. After four days half the cultures were scraped with a sterile microscope slide.

4.107 Plant material

Wheat cv. Monad seeds were sterilised in 100% ethanol for 1 h and then washed 2x in sterile distilled water. The seed coat was removed using sterile tweezers and a scalpel and placed on PDA plates. The grain was then cut into pieces and these were placed on PDA as were entire wheat grains. A range of 10 isolates only were then plated singly on the agar plates and the seeds placed around them. Isolates were then paired (2 isolates per plate) in all possible combinations on PDA. Wheat seeds were placed down the middle of the plate to see if the teleomorph state would form in and around in the seeds. There were four replicates of each treatment.

Wheat leaves that were sterilised in either 0.6% sodium hypochlorite for 10 minutes, 100% ethanol for 10 minutes or autoclaved were placed on moistened filter paper. The 10 isolates were then used singly to inoculate the leaves or in all possible combinations of two isolates. The inoculated leaves were then incubated under near UV light.

4.108 Effects of temperature on growth of *Didymella* in culture

Five media were used in the investigation of the optimum temperature for growth of *Didymella*. The media used were Czapek-dox agar, PDA, Czapek Dox V_8 Barnett and Casamino acid and oatmeal agars. The temperatures tested were 18 and 22 ^{0}C. PDA was used to investigate the effects of different temperatures. The temperatures tested were 10, 14, 18, 22, and 26^{0}C. A range of 16 isolates chosen at random from the culture collection was plated on to the different media with three replicates of each isolate at each temperature. The growth rates of the cultures were measured 24, 48, 72 h after plating. The results were analysed using a generalised linear model. The optimum temperature was then used in all the culturing experiments apart from a selection of media which was incubated for seven days at room temperature then 14 days at a different constant temperature 10, 14, 18, 22, or 26 ^{0}C.

All cultures were examined regularly, approximately every week, and areas of sporulation were identified under a stereomicroscope. A scraping was then taken from the region of sporulation and placed on a microscope slide with a drop of lactophenol cotton blue, and then the slide was examined for the presence/absence of perithecial initials and any developing perithecia under an Olympus BH2 compound microscope. The cultures were examined and the amount of aerial mycelium present on each media type was recorded, as was the abundance of the sporulation of the anamorph state. When cultures appeared old (*i.e.* after 80 days) scrapes were taken of the whole culture and these were then examined under the microscope.

4.109 The use of paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) to detect latent infections by *Didymella* in wheat

Whole plants were inoculated with 1mm by 1mm blocks of mycelia from the edge of three different actively growing *Didymella* isolates. Leaves of varying ages from one week to three weeks were used. Plants were maintained under a high humidity. One week after inoculation, the leaves were detached and cut into two cm long sections. Plastic (9cm) Petri dishes were lined with two layers of sterilised filter paper on the base. The filter paper was moistened with sterile deionised water. Dilutions of 'Gramoxone' (which contains as the active ingredient 200g/l paraquat) were prepared. The concentration used was equivalent to that used to control barley grass (24ul/ml), 2x this concentration (48ul/ml) and ½ the concentration (12ul/ml) required for barley grass. The dilutions were made up to 20ml with sterile deionised water. The leaves were then dipped into the paraquat solution for 1 minute and then placed on the moistened filter paper. The Petri dishes were then placed under near - UV light at 18-20^{0}C and examined every 3 days for the development of pycnidia or perithecia. The filter paper was kept moist during this time with sterile deionised water. At each concentration of paraquat a 1mm section of leaf material was removed and placed on a PDA plate to observe whether *Didymella* was in fact present within the leaf laminar.

4.20 Results

4.201 Growth rate of *Didymella* cultures on PDA at different temperatures.

Of the temperatures tested the radial growth rate of graminicolous *Didymella* isolates on PDA was greatest at 18 and 22^{0}C. At these two temperatures the growth rate was significantly faster than it was at 10^{0}C but not from 14^{0}C. The growth rate at 18^{0}C was also significantly faster than that at 26^{0}C. There were no differences between the growth rates of the different within or between species.

4.202 Growth rate of *Didymella* on Oatmeal, Czapek-dox, Czapek-dox V_8, Barnett Casamino acid and Potato dextrose agars at 18 and 22 ^{0}C

There was no significant effect of temperature on the growth rate of isolates on the different media. The growth rates of the three isolates were significantly different ($p<0.01$) at all temperatures investigated. Growth of all isolates was fastest on CD-V_8 agar. Growth of all isolates was significantly faster on PDA and CD-V_8 than on Czapek-dox, Barnett Casamino acid and oatmeal agars ($p<0.001$)(Figure 4.1). There was no significant difference between the growth rate of isolates on Czapek-dox, and oatmeal agars. Radial growth of *Didymella* was slowest on Barnett Casamino acid agar. All possible interactions (media*isolate ($p<0.05$), media*temperature ($p<0.001$), isolate*temperature ($p<0.01$)) were significant. The growth rate of isolate 1 was significantly greater than that of isolate 3 and 6 at 18^{0}C and 22^{0}C on PDA, Czapek-dox and oatmeal agar.

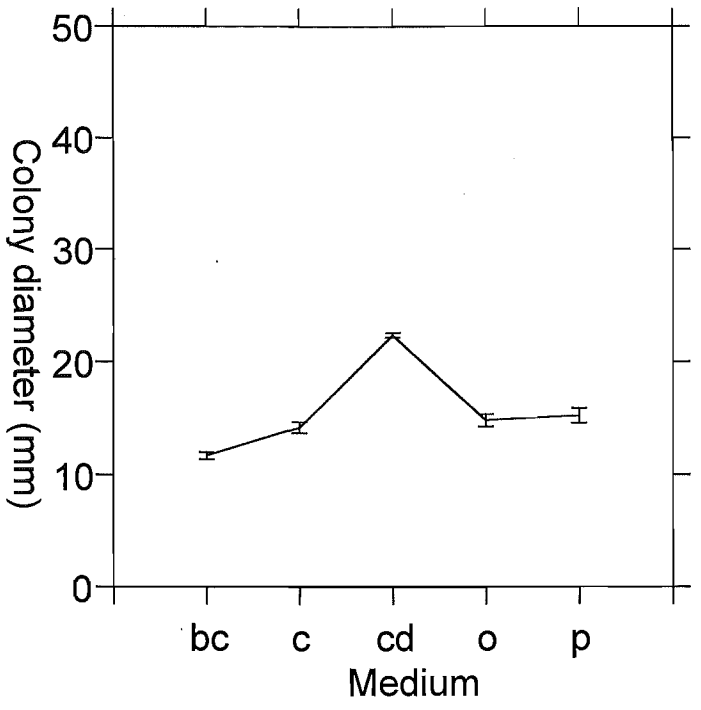

Figure 4.1 The effects of media on the colony diameter of *Didymella* isolates (three replicates per medium were used) at 18^{0}C 48h after plate inoculation. Mean value ± standard error. Medium are: bc, Barnett casamino acid medium; c, Czapek –Dox; cd, Czapek - Dox V_8; o, Oatmeal; p, PDA.

4.203 The effect of pH on the growth rate of *Didymella* in culture

The radial growth of *Didymella* was greatest on PDA amended to pH 5.5-6.5 and was significantly faster than growth on any other pH range media. The radial growth of *Didymella* on pHs' other than 5.5-6.5 ranges were not significantly different from each other.

4.204 The influence of inoculum age on the production of the teleomorph and the anamorph in culture

There was no significant effect on production of the anamorph using inoculum of different ages (data not shown) on any of the media trialed. The teleomorph was not produced in any of the cultures.

4.205 The effect on the level of sporulation of *Didymella* species when different inoculum sources are used

Sporulation of the anamorph was increased and occurred one week earlier on media inoculated with spore suspensions of *Didymella* than when the media were inoculated with a mycelial plug. The teleomorph was not produced in any culture.

4.206 Effect of light conditions

Growth was significantly slower on plates incubated in the dark versus light ($p<0.05$). On PDA and CD-V_8 the level of sporulation of the anamorph was half that of plates incubated under light conditions. On the other media used, dark, light or changing light conditions did not have any effect on the production of the teleomorph. The use of near-UV light encouraged sporulation of the anamorph to a greater level than those cultures incubated under fluorescent light. Sunlight filtered through glass and direct sunlight did not encourage sporulation of the teleomorph.

4.207 Aeration of cultures

The aeration of cultures for five min a day for 14 days reduced the level of condensation on the lids of the plates but did not appear to alter the level of sporulation of any isolate on any of the media tested. Malt extract broth with wheat straw produced more pycnidia on the wheat straw in aerated flasks than in non-aerated where the sporulation was either not present or sparse.

4.208 Mating of isolates

The majority of isolates grew together on the tested media without any line of inhibition. Sporulation levels were the same as observed when the isolates were plated singly. Only four out of 10 isolates showed a line of inhibition but they sporulated as they had when plated singly.

4.209 The use of paraquat for the detection of latent infections and the production of the teleomorph

No sporulation was observed on leaves treated with paraquat. The one mm sections of leaf material that was plated on to PDA were all found to have *Didymella* growing from them showing that infection had occurred. Sporulation of the anamorph was only observed on the untreated control leaves.

4.210 Effect of dehydration on cultures

Two isolates produced the teleomorph state, with immature ascospores as the cultures were drying out. This could not be repeated with the two isolates or any other isolate.

4.211 Media effects

Alphacel medium

Cultural characteristics: rosy buff centre outside ring grey olivaceous. Top: white sparse cottony mycelia.

Formation of locules: none

Level of sporulation: sparse (when wheat straw was added sporulation was abundant)

Formation of the teleomorph state: no

Colouration of conidia: yellow

Observed differences among isolates plated: none

Asthana and Hawker medium A

Cultural characteristics: dark green herbage reverse with fluffy white aerial mycelia on the top

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Barnett Maltose Casamino medium

Cultural characteristics: buff floccose top and reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Browns' medium

Cultural characteristics: salmon underneath with white floccose top

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Carnation leaf agar

Cultural characteristics: grey olivaceous top

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow/hyaline

Level of sporulation: sparse only occurring around leaf pieces

Observed differences among isolates plated: Some isolates had hyaline conidia while others had straw yellow

Czapek-Dox

Cultural characteristics: white to buff cottony mycelia with a buff reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Effects of changing carbon and nitrogen sources on the production of the teleomorph: none

Czapek-dox V-8® juice agar

Cultural characteristics: olivaceous floccose mycelia reverse could not be seen due to the turbidity of the medium

Formation of locules: none

Formation of the teleomorph state: yes, two isolates produced perithecia and ascii, but no ascospores (this could not be repeated).

Colouration of conidia: yellow

Level of sporulation: abundant

Observed differences among isolates plated: apart from the production of the teleomorph state no differences were observed

Cornmeal agar

Cultural characteristics: white cottony mycelia, and white to buff reverse

Formation of locules: yes

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Lima bean agar

Cultural characteristics: white cottony mycelia and buff reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

PDA

Cultural characteristics: variable one of three distinct types (see Chapter 5 for a description of the three types).

Formation of locules: yes

Formation of the teleomorph state: yes, two isolates sporulated on PDA after the cultures had been allowed to dry down

Colouration of conidia: yellow/hyaline

Level of sporulation: abundant

Observed differences among isolates plated: isolates either had straw yellow or hyaline conidia.

Lactose Casein hydrolysate medium

Cultural characteristics: brick red reverse with olivaceous green concentric rings and a grey olivaceous top

Formation of locules: yes

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Leonian

Cultural characteristics: pale luteous top and dark herbage green reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Lilly and Barnett agar

Cultural characteristics: standard medium: buff woolly mycelia on top with dark vinaceous concentric rings on a straw yellow background on the reverse.

0.1% yeast extract: sparse to woolly flat mycelium which was buff in colour with vinaceous buff with a brown vinaceous centre on the reverse
Casamino acid: same as standard medium
Changing glucose and asparagine concentrations: dark vinaceous centre with rosy buff concentric rings with a buff background.
Formation of locules: none
Formation of the teleomorph state: no
Colouration of conidia: yellow
Level of sporulation: abundant except on the medium supplemented with 0.1% yeast extract where the level was sparse
Observed differences among isolates plated: none
Effects of changing carbon and nitrogen sources on the production of the teleomorph: none
Effects of altering glucose and asparagine concentrations on the production of the teleomorph: none

Malt agar (Leonian version)
Cultural characteristics: grey olivaceous top and brick red reverse
Formation of locules: none
Formation of the teleomorph state: no
Colouration of conidia: yellow
Level of sporulation: medium
Observed differences among isolates plated: none

Malt extract broth with and without wheat straw
Cultural characteristics: same as those observed on PDA
Formation of locules: none
Formation of the teleomorph state: no
Colouration of conidia: yellow
Level of sporulation: sparse in aerated cultures with wheat straw; absent in non-aerated cultures and those with no wheat straw
Observed differences among isolates plated: none

Merck PDA.

Cultural characteristics: buff flat mycelium on the top with a buff reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: NA

Level of sporulation: none

Observed differences among isolates plated: none

Milk PDA

Cultural characteristics: black vinaceous top and reverse very dense mycelial growth

Formation of locules: yes

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Mineral peptone agar

Cultural characteristics: buff aerial mycelia with a olivaceous green reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

***Neurospora* complete, *Neurospora* minimal, and *Neurospora* sex synthetic media**

Cultural characteristics: Green olivaceous top and rosy salmon reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow and hyaline

Level of sporulation: sparse but abundant on *Neurospora* sex synthetic medium

Observed differences among isolates plated: none

Oatmeal agar

Cultural characteristics: white to buff mycelia top and bottom

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Potato carrot agar

Cultural characteristics: white to buff aerial mycelia and buff reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: straw yellow and hyaline

Level of sporulation: sparse

Observed differences among isolates plated: none

Potato marmite agar

Cultural characteristics: olivaceous floccose top and olivaceous reverse

Formation of locules: present

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: abundant

Observed differences among isolates plated: none

$^{1}/_{10}$ PDA

Cultural characteristics: white aerial mycelia and buff reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow and hyaline

Level of sporulation: sparse

Observed differences among isolates plated: none

PDA and malt extract and PDA and yeast extract

Cultural characteristics: grey olivaceous floccose mycelia with green and brick red concentric rings on a grey olivaceous background on the reverse of the culture

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow and hyaline

Level of sporulation: abundant

Observed differences among isolates plated: none

Prune agar

Cultural characteristics: white aerial mycelia with a white reverse with a fuscous black centre

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Ross' agar

Cultural characteristics: grey olivaceous floccose

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

Sach's agar

Cultural characteristics: sparse white aerial mycelia

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow and hyaline

Level of sporulation: absent

Observed differences among isolates plated: none

***Septoria nodorum* sporulation agar**

Cultural characteristics: grey olivaceous floccose mycelia with a green olivaceous reverse

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: abundant

Observed differences among isolates plated: the two isolates that produced beaked pycnidia on other medium produced normal shaped pycnidia on this medium. Conidia produced on this medium could germinate unlike the conidia produced on the other media.

Effects of changing carbon and nitrogen sources on the production of the teleomorph: none

Trione mixed cereal grains

Cultural characteristics: sparse white mycelia

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: sparse

Observed differences among isolates plated: none

V_8 juice agar

Cultural characteristics: grey olivaceous floccose mycelium reverse cannot be seen clearly due to the turbidity of the medium

Formation of locules: none

Formation of the teleomorph state: no

Colouration of conidia: yellow

Level of sporulation: abundant

Observed differences among isolates plated: none

Water, Water + $NaN0_3$ and $MgSO_4$**,Wheat leaf decoction and Wheat straw agar**
Cultural characteristics: sparse white aerial mycelium
Formation of locules: none
Formation of the teleomorph state: no
Colouration of conidia: yellow and hyaline
Level of sporulation: sparse
Observed differences among isolates plated: none

Yeast extract agar
Cultural characteristics: Buff aerial mycelium
Formation of locules: none
Formation of the teleomorph state: no
Colouration of conidia: yellow
Level of sporulation: none
Observed differences among isolates plated: none

4.30 Discussion

In nature, some fungi produce large numbers of spores whilst others produce very few; a similar phenomenon can be observed on semisynthetic media. The actual factors that influence sporulation in the laboratory are not well understood (Richards 1951). The conditions required for asexual reproduction and growth are usually wider than those required for sexual reproduction (Lilly and Barnett 1951). The conditions required for growth and asexual reproduction of *Didymella* spp. were investigated as well as the conditions required for the production of the teleomorph in culture.

The consistent production of the teleomorph state of *Didymella* in culture would have provided pure extracts, which could be used for allergy testing, to allow the elucidation of the role of *Didymella* in late summer asthma. The production of the teleomorph and the anamorph in culture would have simplified some of the taxonomic problems associated with *Didymella.* However in this study the teleomorph state could

not be produced regularly, and only immature ascospores were produced within those perithecia which formed.

Many factors have been shown to induce or stimulate the production of perithecia in culture. Actinomycete or bacterial contamination, the use of herbicides, the concentration of carbon and nitrogen and the ratio between them, and flooding the culture with water and then slowly dehydrating the culture on filter paper are a few methods that allowed the production of teleomorphs of other species (Gindrat 1966 in Cunningham 1981, Nilsson 1969 in Cunningham 1981). In the case of *Didymella* accidental contamination with bacteria did not give rise to the production of the teleomorph state. Cultures were also dried down slowly in Petri dishes, which gave rise to the teleomorph state on two occasions; however, this result could not be replicated.

4.31 The effects of temperature, humidity and light on fungal sporulation

Temperature plays an extremely important role in sporulation and affects almost every function of the fungus (Lilly and Barnett 1951). Light, according to most reports, seems to affect reproduction only (Lilly and Barnett 1951). Temperature and photoperiod act very differently during the morphogenesis of the perithecia of different species. Differentiation and maturation of *Guignardia didwelli* ascospores require low temperatures as do other pyrenomycetes (Ross and Hamlin 1962 in Jailloux 1992, Lactoste 1963 in Jailloux 1992, Fayret 1975 in Jailloux 1992). *Didymella* cultures on a representative sample of media were aerated (Petri dish lids were removed every day for 14 days in a laminar flow cabinet and cultures were left for five minutes before the lids were replaced), but the aeration of the plates did not influence the level of sporulation of the anamorph. In contrast Leonian (1924 in Barnett and Lilly 1955) tested the effects of reduced oxygen on pycnidial formation by placing cultures of various Sphaerospsidales in dessicators. Reduced oxygen levels reduced the level of sporulation. The cultures of *Didymella* spp. were not subjected to such low oxygen levels and the Petri plates were not tightly sealed, allowing some gas exchange, which may explain why no effects of aeration were found. Henry and Anderson (1948) showed that the accumulation of ammonia reduces sporulation of *Piricularia oryzae,* and Bright *et al.* (1949 in Barnett

and Lilly 1955) reported that an increase in carbon dioxide reduced the sporulation of *Saccharomyces cerevisiae,* however, it had little or no effect on the sporulation of either the teleomorph and the anamorph of *Didymella.*

The optimal temperature for radial growth of *Didymella* in culture is between 18 and 22^{0}C on PDA. The growth rate is slower at the other temperatures trialed but the growth rates at these temperatures were not significantly different.

Relative humidity had no effect on the numbers of sporangia and sporangiola produced by two strains of *Blakeslea trispora* (Goldring 1936 in Barnett and Lilly 1955). Ternetz (1900 in Barnett and Lilly 1955) found for the formation of apothecia of *Ascophanus corneus* high relative humidity was required. Two species of *Magnusia* varied in their requirements for high relative humidity. High relative humidity was not investigated to see what effect it had on the production of the teleomorph in culture. This may be another avenue of investigation.

4.32 The effect of media

The type of growth medium used affects the level of sporulation. Misra and Hague (1960) stated that sporulation was best on low nutrient media and Punja and Adams (1982) found that 6-8 mm diameter mycelial plugs from an 8-10 day old culture gave the greatest level of sporulation. Graminicolous *Didymella* spp. isolates in this study sporulated best on CD-V_8 agar followed by PDA, two relatively high nutrient media. Starvation did not appear to induce the formation of the teleomorph state as CD-V_8 was the only medium that perithecia were produced on. No ascospores were produced in the asci. Isolates that produced perithecia were plated again on the same medium but without success.

When cultures were placed in the dark, mycelial growth was at the same rate as cultures in the light, however, sporulation was reduced and pycnidia were produced in locules that were embedded in the medium. Low nutrient media gave rise to little mycelial growth and sparse sporulation. Two isolates that previously produced normal pycnidia began to form papillate warty pycnidia on PDA. This was not a result of

repeated subculturing as mycelial blocks from isolates stored in water at four degrees Celsius were used to inoculate all media in this study. This happened on every medium except on *Septoria nodorum* Sporulation Agar where normal pycnidia were produced. According to Scharen and Krupinsky (1971) papillate warty beaked pycnidia are typical of *Ascochyta tritici,* however, as normal pycnidia were produced on *S. nodorum* Sporulation Agar perhaps the warty beaked pycnidia in this study may be due to a mutation and *S. nodorum* agar provides a nutrient that overcomes this mutation.

Holden and Hornby (1981) found for *Gaeumannomyces graminis*, *G. graminis tritici*, *G. graminis graminis* and *G. graminis avenae*, no single in-vitro method regularly produced perithecia. Full strength PDA was found to be the least effective, but when yeast extract was added and the PDA was ¼ strength, it was as effective as malt extract broth for *G. graminis tritici* but not for *G. graminis graminis* or *G. graminis avenae*. The most severely diseased roots tended to be those infected with the fungi that produced the most perithecia.

Due to the tendency of the formation of perithecia to become erratic or cease in cultures maintained in storage for prolonged periods (Davis 1925 in Holden and Hornby 1981, Willets 1961 in Holden and Hornby 1981) cultures in this study that had remained in storage for longer than six months were not used in the attempts to produce the teleomorph state.

McOnie (1964) found that *Gnomonia fucticola* (Arn.) Fall., the causal agent of strawberry leaf scorch, was stimulated by an increased concentration of conidia in the spore suspensions used to inoculate cultures. This was tested for *Didymella* and while it did lead to an increased level of sporulation it was only of the anamorph. Further work (McOnie 1964) investigated the use of vitamins to replace the high concentration spore suspension but only the spore suspension was successful in inducing increased sporulation of *G. fruticola.*

Ross (1961) investigated the use of chemical elements on the formation of perithecia of *Venturia inaequalis*. Nitrogen was the only element that had an effect at

low concentrations. In media containing low nitrogen no perithecia were produced. The nitrogen source and the cation present in the salt also influenced the production of perithecia. The perithecial production of *Melanospora destruens* Shear (Hawker 1939 in Ross 1961) decreased as the concentration of glucose increased above 0.5% and was entirely inhibited at 2%. Westergaard and Mitchell (1947 in Ross 1961) found that high concentrations of potassium nitrate inhibited the initiation of prototheca of *Neurospora crassa* and that complete exhaustion of nitrate had to occur before the prototheca developed into perithecia. The teleomorph of *V. inequalis* is produced on overwintering leaves where presumably the nutrient levels drops as the formation of perithecia occurs (Ross 1961).

Buston and Basu (1948 in Hawker 1951) showed very low sugar media which was high in thiamine promoted very little mycelial growth but encouraged the development of perithecia. It was therefore hypothesised that perithecia would be produced if available hexoses were maintained at low levels. The formation of perithecia on medium containing 2% glucose or sucrose was tentatively explained by Bretzloff (1954 in Ross 1961), who found that immature perithecia were produced but they were aborted in the presence of 2% hexose sugar. Media containing differing levels of both nitrogen and different nitrogen sources and carbon and different carbon sources were tested without the production of the teleomorph in any instance. Abundant aerial mycelium was produced in cultures with a high glucose level.

The optimal pH range for most fungi is 5-6 (Lilly and Barnett 1951). *Didymella* grew best on a medium at a pH of 5.5-6.5 and the level of sporulation was similar to isolates grown on PDA. Growth at other pHs' were not significantly different from each other. The isolates used in the investigations into growth at different temperatures and the effect of media were generally not significantly different from each other apart from two isolates. Chi and Hason (1964) found that the optimal pH for the growth of *Fusarium roseum, F. oxysporum* and *F. solani* was 5.0-5.5. No evidence of two optimum pH ranges was obtained (Lilly and Barnett 1951).

The addition of activated charcoal to a variety of media was trialed by Martinez-Espinoza *et al.* (1992) and was found to improve stability and strength of the mating reaction of *Ustilago hordei*. Activated charcoal-amended media had no effect on *Didymella* sporulation. When *Didymella* cultures were paired they generally grew into each other and there was no line of sporulation at the point where the isolates met. The sporulation level of each fungus was the same as it was in an individual culture.

Paraquat has been used to detect latent infections by fungi in various hosts (Cerkauskas *et al.* 1979 in Sinclair and Cerkauskas 1996, Cerkauskas and Sinclair 1980 in Sinclair and Cerkauskas 1996, Hodges 1981, Cowling *et al.* 1984 in Sinclair and Cerkauskas 1996). It was used as field observations had shown that *Didymella* sporulated within lesion created by paraquat spray drift. It was suggested by Sinclair and Cerkauskas (1996) that, after application of paraquat, nutrients are released from plant cells which may affect the latency of the fungus by stimulating appressorial germination or the growth and development of subcuticular hyphae. (Muirhead 1981, Muirhead and Deverall 1981, Simmonds 1963). *Didymella* did not sporulate on leaf tissue that had been treated with paraquat whereas the control leaves that were inoculated with *Didymella* only, sporulated under near- UV light. *Didymella* could be recultured from the leaves prior to treatment with paraquat. Wheat leaves that had been sprayed with paraquat via spray drift in the field produced pycnidia in the laboratory when incubated under near-UV light.

Didymella spp. are very common sporulating on senesced leaf tissue in the field (Cromey *et al.* 1994b) but no consistent production of the teleomorph in the laboratory could be achieved. In the laboratory approximation of the photoperiod, light wavelengths, temperature and humidity fluctuations occur. It appears in the case of *Didymella* spp. that the sporulation of the teleomorph state requires a yet unknown combination of many of these factors for sporulation. Biotic stresses may also play a part, perhaps plant stress, as a result of infection by a pathogen or an attempted infection by a saprophyte triggers *Didymella* to start ramifying through the host leaf tissue and then sporulate. To obtain a more accurate idea of the abiotic conditions in the field data loggers could be set up to obtain temperature, humidity, rainfall data which may allow

the conditions required for the production of the teleomorph in vitro to be defined. Inoculation of plants already infected with *Didymella* spp. with selected wheat pathogens and saprophytes could also be attempted to see if this has any effect on the formation of leaf scorch and the production of the teleomorph.

In summary the radial growth of the *Didymella* isolates in this study was fastest on CD-V_8 agar at between 18 and 22 ^{0}C. Radial growth of graminicolous *Didymella* spp. was fastest on PDA that had a pH range of 5.5-6.5. Every fungus differs in its requirement for nutrients, light and temperature for sporulation and for many fungi the exact conditions are unknown. *Didymella* readily produces the anamorph in culture, the production of the teleomorph in vitro remains elusive.

5.0 IDENTIFICATION OF GRAMINICOLOUS *DIDYMELLA* SPECIES IN NEW ZEALAND

5.01 The taxonomy of *Didymella* species

Summary tables of the published descriptions of *Didymella* species and their *Ascochyta* anamorphs are given in Tables 5.1 and 5.2, where it can be seen that the diagnostic differences between some of the species are small. In some cases the production of the teleomorph or the anamorph is required to identify a particular isolate. For example, to distinguish between *D. exitialis* and *D. autumnalis* the anamorph must be produced and examined (Punithalingam 1979b). Allitt (1986) in a study of airborne ascospores, assigned roughened ascospores to *D. exitialis,* but the descriptions published by Punithalingam (1979a) stated that *D. exitialis* has smooth ascospores. Within sets of airborne ascospores the size and the number of guttules varied and Allitt (1986) suggested that this was due to the different maturities of the ascospores. Cromey *et al.* (1994a) recorded *D. exitialis* on wheat and Riesen (1987) recorded *D. phleina* on barley. On the application form for the submission of Riesen's isolate of *D. phleina* to the Plant Diseases Division Culture Collection, he stated that the anamorph state had been identified by Punithalingam as *A. avenae,* however the teleomorph produced in culture proved to be *D. phleina.* This indicates the difficulties in identifying *Ascochyta* species. Mace (1994 unpublished) found difficulties in identifying the different species by conidial dimensions and shape, as the shapes of conidia were very variable; within a single spore isolate conidia fitted the descriptions of five different species.

Table 5.1 Published descriptions of Graminicolous *Didymella* species

Perithecia	Asci	Ascospores	References and Remarks
***Didymella exitialis* (Morini) Müller** On wheat leaves immersed dark brown black subglobose, small papillate ostioles. Pseudoparenchymatic cells several layers thick make up the pseudothecial wall. Outer cells are heavily pigmented **Diameter: 90-130µm**	Numerous, Cylindrical to subclavate or broadly obovoid, straight and arranged in a relatively flat layer. Ascus wall bitunicate and thick and ascus contains eight ascospores. **40-60 x 8-12µm**	Hyaline, guttulate, medianly uniseptate approximately, constricted slightly at septa, ellipsoid or slightly biconic and irregularly biseriate. **12-14 x 4-5.5µm**	Punithalingam (1979a) Causes leaf scorch of *Hordeum vulgare* and *Triticum aestivum.* Abundant in Britain on wheat and barley leaves. Airborne ascospores have been reported to have a role in late summer asthma
Diameter: 100-150µm	**46-66 x 8-12µm**	'rather strongly tapered and often slightly bent' **13-16 x 2.5-4µm**	Müller (1952)
		16.5-20 x 4.0-5.0 (5-7)µm	Allitt (1986) observed in culture on oat agar from ascospores collected from airspora
Didymella phleina Punith. & Kåre Årsvoll sp. nov. Pseudothecia produced on oat agar along with pycnidia, subglobose and ostiolate, colour ranging from yellowish brown to chestnut brown. Pseudothecial wall composed of layers of cells 2-3 thick **120-140µm**	Almost sessile, arranged in an approximately flat layer straight and cylindrical, thick bitunicate ascus wall, pseudoparaphyses septate and filiform noticeable in immature pseudothecia **50-70 x 10-12µm**	Ends acute, medianly uniseptate, guttulate, fusiform to ellipsoid and distichous **16-17 (18) x 4-5µm**	Punithalingam (1979b) Isolated from *Bromus carinatus, Hordeum vulgare, Melica altissima, Phleum pratense, P. phleoides* and *Lolium* sp. (Arsvoll 1975) The fungus attacks severely hardened and nonhardened plants
100-150µm	**50-60 x 10-12µm**	**15.5-18.5 x 4.5-5µm**	Riesen (1987) Shape of the ascospores was similar to *D. exitialis*
		Ends are not acute and both roughened and smooth ascospores present in airspora **19.0-22 x 5-6.5 (5.5-7.5)µm**	Allitt (1986)
***Didymella graminicola* Punith. sp. nov.** Pseudothecia formed on potato dextrose agar scarce and not immersed in the medium. Culture appears grey brown eventually turning violet on potato dextrose agar. Pale yellow brown angular cells make up the pseudothecial wall in layers of 2-5 cells thick, dark pigmented cells line the ostiole. **160-200µm**	Numerous, present a relatively flat layer, straight cylindrical to subclavate, thick bitunicate ascus wall. Ascii are eight spored and pseudoparaphyses are noticeable in young fructifications and are filiform septate and sometimes branched **40-50 x 10-12µm**	Ovoid to elliptical, 1 septate constricted at the septum, lower cell is slightly smaller than the upper, irregularly distichous **15-18(20) x 3-4(-5)µm**	Punithalingam (1979b) isolated from *Lolium* seeds
***Didymosphaeria loliina* Punith. sp. nov.** On oat agar the colony is grey brown with dull white patches and is colourless underneath with black spots. Brown vinaceous chlamydospores. Pseudothecia have short necks, are ostiolate and fuscous black in colour. Two –4 layers of pseudoparenchymatic cells make up the pseudothecial wall **220µm**	Numerous, straight, subclavate or broadly obovoid 8-spored ascii arranged in a relatively flat layer. Ascus wall is bitunicate and thick. Septate and filiform pseudoparaphyes are present and more noticeable in young fructifications. **40-70 x 10-15µm**	Guttulate, medianly uniseptate, broadly ellipsoid or slightly biconic and hyaline in colour later turning brown **10-16 x 3.5-5µm**	Punithalingam (1979b)

Table 5.2 The published descriptions of Graminicolous *Ascochyta* species.

Pycnidia	Conidiogenesis cells and Conidia	References and Remarks
Ascochyta* state of *Didymella exitialis Culture on oat agar. Reverse is fuscous black. Floccose and greyish sepia to fuscous black with white or buff blotches. Loosely aggregated groups of pycnidia can be up to 800µm wide. Pycnidia are yellowish brown, subglobose and are immersed and become erumpent. Thin walled, hyaline pseudoparenchymatic cells 2-3 cell layers thick make up the inside pycnidial wall. On the outside are heavily pigmented thick walled cells. In culture the wall is stromatic and composed of several layers of thick-walled pseudoparenchymatic cells **120µm** ostioles nearly rounded **10-20µm**	Conidiogenesis cells are phialidic, somewhat doliform and hyaline. Conidia are hyaline, medianly septate with no constriction at the septa, ellipsoid, base is sometimes flat or more usually the base and the apex are rounded, narrowed gradually at the apex, and guttulate. conidia on oat agar are narrower but similar to those produced on the host **15-18 x 3.5-4µm**	Punithalingam (1979b)
	12-23 x 3-4µm	Müller (1952)
	15.0-18.0 x 3.5-4.0µm (on wheat straw)	Allitt (1986)
***Ascochyta phyllachoroides* Sacc. &Malbr. Forma *melica* Fautrey** The colony is initially white on oat agar. In appearance it is floccose to felted. The reverse turns dark brick and the top, grey olivaceous over time. Yellowish brown to dark brick/black pycnidia which are globose and ostiolate. Two to 4 layers of pseudoparenchymatic cells make up the pycnidial wall **≤250µm**	Hyaline, phialidic and doliiform conidiogenesis cells. Conidia range in colour from very pale straw yellow to pale straw yellow and the wall is smooth to finely roughened. The conidia have either flat or rounded base and a rounded but sometimes narrowed apex. Guttulate, oblong to broadly ellipsoid and medianly uniseptate. **18-20(-24) x 5.5-6.5(-8) µm**	Punithalingam (1979b)
Pycnidial wall was yellow-brown and composed 3-5 layers of pseudoparenchymatic cells. The inner layer was hyaline as were the phalides. **150-290µm**	Conidia were straw yellow and fitted the shape description of Punithalingam. In older cultures the wall was roughened **16-21 x 5-6 µm**	Riesen (1987) Produced on Malt extract agar
	18.0-20.0 (-24) x 5.5-6.5 (-8.0)µm	Allitt (1986)
***Ascochyta hordei* Hara** Causes buff to white rounded leaf spots 4-10mm in diameter reddish brown margins. Cultures appear floccose and are white buff in colour and the reverse is sepia to dark mouse grey. Pycnidia in culture are yellow brown and are not abundant. Pycnidia on a host are pale luteous to ochraceous in colour. Pycnidial wall is made up of 2-3 layers of thin isodiametric cells of which the innermost layer is hyaline. The cells lining the ostiole are darker in colour than the rest of the outer wall. Pycnidia are subglobose immersed but become erumpent. **≤160µm** **≤200µm in culture, ostiole is ≤30µm**	Conidiogeneous cells are doliiform to phialidic. The first conidia are produced by temporary holoblastic conidiogeneous cells which are slough off as the pycnidium matures Pale yellow, medianly uniseptate rarely 2-septate, cylindrical to ellipsoid with slight narrowing at the apex. Not guttulate usually. conidial wall smooth to finely rough and thin. **17-20(-22) x 3.5-4(-4.5)µm**	Punithalingam (1979b)
Thin walled pycnidia, spherical to subspherical in shape, ostiolate and erumpent **100-172µm**	One septate, rarely two, cylindrical with rounded ends or sometimes slightly twisted but not strongly tapering to either end (except on *Holcus* and cultivated barley), hyaline to very pale yellow in colour. **16-22 x 4.8-6.2µm** (*Festuca* sp. & *Hordeum murinum* **17.8-21 x 5.3-7.1µm** *(Bromus)* **18-27 x 5.5-6.8µm** (*Holcus* and cultivated barley United States of America) **17-28 x 4-6µm** (cultivated barley Japan modified from Hara (1916)	Sprague (1950)

Table 5.2 Cntd The published descriptions of Graminicolous *Ascochyta* species.

***A. hordei* var. *europea* Punith. Var. nov.** Yellowish brown **100-180μm**	Hyaline to pale buff **14-16 x 3-4.5 (-5)μm**	Punithalingam (1979b)
***A leptospora* (Trail) Hara.** Yellowish brown to rust brown **140-220μm**	Cylindrical and of uniform width **14-16(18) x 2.5-3μm**	Punithalingam (1979b) Punithalingam (1979b)
***Ascochyta* state of *Didymella graminicola* Punith.** Dark brown to black pycnidia. Two -5 layers of pseudoparenchymatic cells somewhat darker towards the ostiole and the neck make up the pycnidial wall. Pycnidia are either single or aggregated and are spherical to subglobose. **120-180μm**	Conidiophores arise from undifferentiated cells in the pycnidial lining. They are short and obpyriform 6-10 x 6-8μm Straight, 1- septate (not constricted at the septum) cylindrical to ovate and hyaline conidia **14-18 (-20) x 3-4μm**	Punithalingam (1979b)
***Ascochyta* state of *Didymosphaeria loliina* Punith Smedgaard - Petersen. Nov.** Subglobose, ostiolate, hazel brown and present in culture either singly or aggregated and are numerous. Two to four layers of pseudoparenchymatic cells make up the pycnidial wall. **140-200μm**	Temporary and permanent conidiogenous cells. Both are hyaline. The temporary are holoblastic while the permanent are somewhat doliiform and phialidic. Conidia are rounded at the ends, cylindrical, ellipsoid or oblong, occasionally 2-septate usually medianly uniseptate with no constriction at the septum and guttulate. **8-12(-14) x 2.5-4μm**	Punithalingam (1979b)
***A. hordeicola* Punith. sp. nov.** Orbicular leaf spots which are vinaceous buff to fawn with a purple brown margin 2mm-1cm. Pycnidia are yellowish brown, immersed becoming erumpent and globose. The pycnidial wall is 2-4 layers of pseudoparenchymatous cells thick of which the innermost is hyaline. The ostiole is rounded and the cells are fawn coloured. **140-200μm** **ostiole 15-20μm**	Conidium is narrowed towards the apex, which is rounded, and there is no constriction at the septum. Base is either flat or rounded. Cylindrical to fusiform in shape with guttules. Conidia are hyaline. **14-16(-20) x 3.5-4μm**	Punithalingam (1979b) Species is a mild pathogen of barley and wheat. Common on wheat in Glamorganshire (Roberts in Punithalingam 1979b)
***A sorghi* Sacc.** Rust to sepia in colour, stromatic, subcuticular or immersed. Innermost layer of pycnidial wall is hyaline. **≤200μm**	Predominantly uniseptate **16-20(-21) x 6-8μm**	Punithalingam (1979b)
Golden-brown with darker cells just below the ostiole, walls range from soot black to a paler shade. Erumpent and subglobose **90-140μm**	Fusoid, subcylindrical to ovate. **11-21 x 1.6-4.0μm**	Sprague (1950) North America, Asia, Europe, Germany, Czechoslovakia, Greenland, Japan, Poland, India, Scotland, Turkey, Uruguay and Russia
***A. graminicola* Sacc.**		Punithalingam (1979b) examined and excluded the species from the genera *Ascochyta* and in many cases has been found to be *Darluca filum* (Biv.) Cast.
		Sprague (1950) assigned the species to *A. sorghi*.
Dark brown **100-150μm**	One septate spores, oval or spindle shaped with tapered ends **10-14 x 3-4μm**	Hara (1916 in Punithalingam 1979b)
***A. graminis* Sacc.**		Punithalingam (1979b) Examined and excluded from *Ascochyta*

Table 5.2 Cntd The published descriptions of Graminicolous *Ascochyta* species.

***A. avenae* (Petrak) Sprague and Johnson.** Pycnidia are yellow brown with a small papillate ostiole, which breaks through the epidermis. Pycnidia are globose and the ostiole is rounded and the cells surrounding it are darker than the pycnidial wall. The wall is made up of pseudoparenchymatic cells 2-3 layers thick. The innermost cells are hyaline. **140-190µm** **ostiole diameter ≤30µm**	Permanent conidiogeneous cells are hyaline, mostly doliiform and hyaline. Conidia somewhat ellipsoid, the base is slightly narrowed but flat or rounded, gradually narrowed towards the apex. Apex is rounded, nearly uniseptate, spores are sometimes guttulate and the walls are smooth **17-20 x 6 (-7)µm**	Punithalingam (1979b) On *Avenae sativa, Helictotrichon mortonianum, Hordeum vulgare, Lolium perenne* and *Triticum* sp.. Causes leaf spot on cereals and grasses but the significance has not been determined
Dark brown, ostiolate wall is 5-10µm thick **100-200µm**	Conidia have rounded ends, are irregularly cylindrical to fusoid, 1 to 2 celled and yellow in colour **17-26 x 6.2-7µm**	Based on the technical description of Petrak (1925 in Punithalingam 1979b)
***A. avenae* (Petrak) Sprague and Johnson var. *confusa* Punith** Buff to fawn coloured leaf spots with brown vinaceous borders of up to 1 cm in size. The spots are elliptical or irregular. Pycnidia are immersed, becoming erumptant, subglobose and yellow brown in colour. The ostiole is almost rounded. The pycnidial wall is composed of 2-3 layers of pseudoparenchymatic cells. Cultures are floccose, vinaceous buff with blotches ranging from white to yellow at the margins and the base is vinaceous buff on oat agar. Hyaline to chestnut brown mycelium. Pycnidia are yellow brown to isabelline or umber brown. **160-200µm, ostiole ≤20**	Conidiogenous cells are permanent, hyaline, phialidic and ampulliform. Conidia are medianly uniseptate sometimes 2-septate or unequally septate, cylindrical to ellipsoid, with a rounded apex and a truncated or rounded base. Conidia are guttulate. Hyaline to pale yellow in colour. In culture temporary conidiogenous cells exist and these are hyaline, globose and holoblastic **(-15) 18-21 (-24) x 4-5µm**	Punithalingam (1979b) On leaves of *Avena sativa* (cv. *Maris quest*)
***A. desmazieri* Cav.** Elliptical to irregular reddish brown leaf spots with brick red margins ranging in size up to 6mm wide. Yellowish brown, immersed, subglobose and with a rounded ostiole which has dark brown pseudoparenchymatic cells bordering it. Two to 4 layers of pseudoparenchymatic cells make up the pycnidial wall, the inner cells are hyaline while the outer are yellow brown. **140-200µm** **ostiole diameter 10µm**	Permanent conidiogenous cells are cylindrical, hyaline and phialidic. Conidia are cylindrical in shape and two -3 % of conidia are 2-celled or rarer 3. The apex is rounded and the base either truncate or rounded and the overall shape is approximately cylindrical. **14-20(-22) x 2-2.5µm**	Punithalingam (1979b) On *Lolium perenne* and *L. multiforum* leaves
Brown, immersed, compressed globose, more or less grouped, ostiolate. **130-200µm**	Narrowly cylindrical and 1-septate **13-20 x 2-3.5µm**	Sprague and Johnson (1950)

5.02 Taxonomic Analysis

After morphological and molecular characteristics of fungal isolates are determined then analysis is required. There are many different viewpoints on the types of analyses that should be used. Luttrell (1977 in Seifert *et al.* 1995) stated that "taxonomic concepts and classifications are hypotheses." There are no prescribed rules on how these hypotheses are created. Whether the hypothesis is generated by the statistical output from a computer program or an experienced mycologist makes it no more scientific or repeatable (Seifert *et al.* 1995).

Deoxyribose nucleic acid (DNA) sequence data is kept quite separate from other classes of data and the question that Tehler (1995) raises is whether classes of data exist in nature, and if they do then more separation of certain data classes needs to occur. This would give rise to for example chemical data and anatomical data. More explanatory power is achieved by analysing all the characters for congruence (Anderberg and Tehler 1990 in Tehler 1995).

5.03 Molecular and Morphological Taxonomy

The increased use of molecular techniques in mycology has led to major debates between traditional taxonomists and molecular taxonomists, with each group often not understanding the methods of the other (Mitchell *et al.* 1995). Today, many scientists are working towards greater taxonomic understanding by using both methods. Seifert *et al.* (1995) stated that neither sequence nor morphological based taxonomic studies were superior from the perspective of the scientific method. The reproducibility of DNA sequence data is seldom rigorously tested but the observations are considered to be correct. Molecular techniques have opened up areas of research that were once unrealisable due to the lack of effective taxonomic characters. The search for new characters is continuing due to several factors; these are the interdependence of many taxonomic characters, for example, ascus length and ascospore size and the effects of cultural conditions on conidial ontology. This does not mean that characters such as DNA are not subject to any pressures due to environmental conditions. As yet we have no real understanding of the rates of change in the areas of DNA not being studied or which pressures are directing genomic change.

The easiest traits to investigate when identifying a fungus, are morphological traits. The inclusion of molecular information can provide a more accurate picture as to the identity of the fungus and thus this approach has been called phylogenetic character mapping (Mitchell *et al.* 1995).

Traditional methods, for example spore measurements and general morphological characters, have, in some genera, proved to be unreliable. One such

example is the work carried out by Wehmeyer (1927). He studied *Diaporthe* species with anamorphs in the genus *Phomopsis* and his conclusions were that "...the size, shape and structure of the pycnidial stroma, pycnidial locule, and so called pycnidial wall are by no means constant and are very unreliable as diagnostic characters at least in *Phomopsis* and related form genera."

Random amplified polymorphic DNA (RAPD) is a technique that is currently being used to differentiate genetic variation within a fungal population (Tommerup *et al.* 1995, Möller *et al.*, 1995). The method is less time consuming than Restriction length polymorphic DNA (RFLP) but comparable results are obtained which can be analysed in similar ways (Williams *et al.* 1991). The method is based on the amplification using polymerase chain reaction (PCR) of random DNA fragments with short primers of arbitrary nucleotide sequences. Various studies have indicated that this method is very useful for genetic analysis among closely related strains, species or isolates (Welsh and McCelland 1990 in Raina *et al.* 1997, Clark and Lanigan 1993 in Raina *et al.* 1997, Macpherson and Gajadhar 1993 in Raina *et al.* 1997, Williams *et al.* 1993 in Raina *et al.* 1997).

5.04 Aims

To establish the identity of the *Didymella* isolates collected in New Zealand from graminicolous hosts and also observe which species are present on which host.

5.10 Materials and Methods

5.101 Sources of isolates

Isolates used in identification of graminicolous species are given in Appendices three and four.

5.102 Storage of isolates

Isolates were stored either as single conidium or single ascospore isolates at 4^0C or at -80^0C. The isolates, as plugs of mycelium, were stored in 16ml of sterile deionised water in McCartney bottles at 4^0C or in 20% glycerol at -80^0C.

5.103 Culturing

All isolates were cultured on potato dextrose agar (Gibco PDA) plates. Plates were incubated at 20 ^{0}C with a 12 h photoperiod. After seven days incubation, the cultures were scraped with a sterile microscope slide and transferred to near - UV light (Philips Black light) with a 12 h photoperiod and a temperature range of approximately 18 - 20^{0}C, to encourage sporulation. The cultures were described using 'A Mycological Colour Chart' (Rayner 1970).

5.104 *Didymella* species.

Leaf material from wheat (*Triticum aestivum* L.)[80 isolates], barley (*Hordeum vulgare*)[20 isolates], ryegrass (*Lolium perenne* L.)[2 isolates], barley grass (*Hordeum murinum* L.)[4 isolates], cocksfoot (*Dactylis glomerata* L.)[4 isolates], Yorkshire fog (*Holcus lanatus* L.)[2 isolates], prairie grass (*Bromus willdenowii* Kunth)[2 isolates] and wild oats (*Avena fatua* L.)[4 isolates] was examined for pseudothecia and pycnidia of *Didymella* spp. Pseudothecia and pycnidia, when found, were picked off the leaf material using a scalpel blade and mounted in lactophenol cotton blue or lactophenol on a microscope slide. The slide was then examined under an Olympus BH2 microscope using an eyepiece micrometer. Teleomorph characters examined were: pseudothecial diameter, ostiole diameter, ascus length and width and ascospore length and width. For the anamorph, the dimensions measured were: pycnidial diameter, conidial length and width. Twenty to 50 measurements of each character were obtained, depending on the variability of the sample. Colouration of the pseudothecia, pycnidia, ascospores and conidia were noted, as was the shape of the ascospores and the conidia.

5.105 Cultures of *Ascochyta* species.

Single ascospore cultures of *Didymella* species were obtained from wheat [61 isolates], barley [17 isolates], phalaris (*Phalaris aquatica* L.) [5 isolates], prairie grass [10 isolates], cocksfoot [2 isolates], Yorkshire fog [4 isolates], barley grass [3 isolates], ryegrass [2 isolates] and wild oats [7 isolates] from different regions of Canterbury. Single ascospores isolations were made by removing a small area of leaf tissue with

between two and six perithecia. A small dot of vaseline was placed onto the lid of a water agar plate and the leaf tissue was placed perithecia side up. The lid was then replaced on the plate and the plate was placed 45° to the horizontal. The plates were examined after eight hours for discharged ascospores. The ascospores were removed singly from the plate using a sterile scalpel and placed on a PDA plate. The plate was then incubated at 18-20°C and the identity of the isolates as an *Ascochyta* was confirmed.

5.106 Taxonomy of *Ascochyta* species in culture.

Slides were made of representative isolates of the *Ascochyta* state(s) (*Didymella* in culture) isolated during the 1993-97 cereal growing seasons. The specimens were mounted initially in lactophenol so that the colouration of the conidia and the pycnidia could be recorded. For ease and accuracy of measuring conidial length and width slides were then stained with lactophenol cotton blue. Twenty - 50 measurements of each character were made, depending on the variability of the isolate. Characters measured were pycnidial diameter, ostiole diameter, and conidial length and width. Conidial shape was also recorded, as were colony characteristics.

5.107 Taxonomic analysis

Separate analyses were carried out on the teleomorph and the anamorph data. In all analyses, mean values were used, and the published descriptions of graminicolous *Didymella* spp. and graminicolous *Ascochyta* spp (Punithalingam 1979b) were included in the analyses. The distance measure used in the analyses was the normalised euclidean distance (root mean squared distance). The average linkage method was used (Sokal and Michener 1958 in SYSTAT® 7.01 1997).

Hierarchical trees were constructed from the raw data. Hierarchical trees are formed by the calculation of the average Euclidean distance between pairs of observations, or clusters, and merging the two closest into one. Merging is repeated until one cluster is left. The linkage of objects is shown as the joining of branches in a tree.

The linkage of all clusters into one group is the "root" of the tree (SYSTAT® 7.01 1997).

Additive trees were carried out on a dissimilarity matrix of the teleomorph and the anamorph data within SYSTAT® 7.01. Sattath and Tversky (1977 in SYSTAT® 7.01 1997) developed additive trees for modelling similarity/dissimilarity data. Additive trees, unlike hierarchical trees, do not imply that all within-cluster distances are smaller than all between-cluster distances or that within cluster distances are equal. The model is in the shape of a tree with the distances between the objects being represented by the branch length connecting the tree (SYSTAT® 7.01 1997).

Principal components analysis (PCA) was performed using factor analysis in SYSTAT® 7.01. Basically, PCA is the "conceptual flattening of the points in multidimensional space so that they lie on a plane surface" (Abbott *et al.* 1985). A dissimilarity matrix of the teleomorph and the anamorph data was used to calculate principal component scores. An eigenvalue, associated with each component, represented the variance of the component. For each principal component, an eigenvector was calculated, showing the loading on each variable used to generate the principal components scores.

Discriminant analysis was performed using SYSTAT® 7.01. Discriminant analysis is related to multiple regression and multivariate analysis of variance. It can be used to investigate the differences among groups (which are most alike and most different) and the variables that are the most useful at distinguishing among the groups. Isolates were assigned to a species level using the hierarchical and additive trees and the PCA as no known group membership existed. Linear and quadratic functions of the variables are calculated to separate cases into two or more predefined groups. The variables in the linear function were selected automatically by SYSTAT® using backward or forward stepping. The variable that contributes most to the separation of the groups is entered by SYSTAT® at each step for backward stepping.

5.108 Molecular investigation

5.1081 Clamped homogenous electric field (CHEF)

Potato dextrose broth (Difco) was inoculated with mycelial blocks of *Didymella* spp. The broth cultures were then shaken on a rotary shaker for 3 days at 18-20 ^{0}C. Mycelium was then harvested through sterile muslin and mycelium was placed on sterile Petri dishes and placed in a -80^{0}C freezer for 2 hours and then placed in a freeze dryer for 48 hours. Freeze dried mycelium was then stored in dry sterile universal bottles in a dessicator until required. The method of Guidet and Langridge (1994) for preparing yeast chromosomes for pulsed field electrophoresis was used to prepare fungal chromosomes of *Didymella* spp. Four isolates were selected and 1, 10, 50 and 100 mg of dried mycelium was weighed out for each isolate. The mycelium was then crushed in a mortar and pestle under liquid nitrogen. The resultant powder was warmed to 40^{0}C in the pestle and mixed with 2 ml of 1.4 % low melting point agarose (Seakem) in 1 x TE (1mM Tris- HCl, pH 8.0, 50mM EDTA) also at 40^{0}C. The mixture was transferred via Pasteur pipette into a Bio-Rad mould (Richmond, CA) and was allowed to set for 20 minutes at 4^{0}C. Lysis solution was made up containing 10mM Tris-HCl, pH 8.0, 500mM EDTA, 1% Sarkosyl^{TM} , 1 mg/ml proteinase K. The proteinase K was later substituted for either 16 or 20 mg of Novozyme or yeast lytic enzyme in later experiments. The agarose plug was placed in a sterile eppendorf tube in 2 ml of lysis solution at 55^{0}C in a water bath for 1 hour. After 1 hr the plug was washed twice with 1mg/ml proteinase inhibitor. This step was added because degradation of the chromosomal DNA occurred at this stage. The plug was rinsed 3-4 times with 1x TE. A 1% agarose gel in 0.5x TBE buffer was then prepared and the plugs cut to yield a 2-3mm piece which was loaded into the well and sealed with 1% molten agarose. The gel was run in 0.5x TBE for 48 hours. The electrophoresis run was at 200 V with time ramp 50-90 seconds. The gel was then stained with eithidium bromide and examined.

5.1082 Extraction of DNA (Lee and Taylor 1990 with modifications)

Twenty to 60 mg of dried mycelium that had been ground in a mortar and pestle under liquid nitrogen, was placed into an eppendorf tube. Lysis buffer (400 µl) (50mM Tris -HCl, pH 7.2, 50mM EDTA, 3% SDS and 1% 2- mercaptoethanol) was added, and

the mixture vortexed until homogeneous. The mixture was incubated at 65^{0}C for 1 hr. RNAse A was then added for a 10 min digestion followed by a phenol extraction and isopropanol precipitation to remove contaminating RNA. An equal amount of phenol: chloroform: IAA (24:24:1) mixture was added and the tubes were vortexed gently. The tubes were microcentrifuged at 10,000 x *g* at room temperature for 15 minutes. The aqueous phase containing the DNA was removed from the tube and placed in a new eppendorf to which 10 µl of 3 M NaOAc was added. This was followed by 0.54 volumes of isopropanol and the tube was then inverted to mix. The tubes were microcentrifuged at 4^{0}C for 2 min and the supernatant was poured off and the pellet washed once with 70% ethanol. The tubes were then inverted and drained on a paper towel until the plug was dry. The pellet was resuspended in 100 µl of sterile water.

5.1083 The use of DNAzole^{TM} Reagent (Life Technologies Gibco) to isolate DNA of *Didymella* species.

Fifty milligrams of freeze-dried mycelium were ground in mortar and pestles under liquid nitrogen. The powder was then transferred to an Eppendorf tube to which 1 ml of DNAzole™ was added. The eppendorf was centrifuged at 10,000 x *g* at 4^{0}C for 10 min. The supernatant was transferred to a fresh tube. The DNA was precipitated from the lysate by the addition of 0.5 ml of 100% ethanol. The sample was then mixed by inversion and the tubes were stored at room temperature for 3 minutes. After this time, the tubes were centrifuged at 1,000 x *g* for 1-2 minutes at room temperature to pellet the DNA. The pellet was then washed twice with 1 ml of 95% ethanol and the tubes were inverted 3-6 times. The ethanol was removed using a pipette. The DNA was dried by inverting the open tubes and storing them at room temperature. After the pellets were dry, 0.8 ml of sterile double distilled water was added and the pellet resuspended.

5.1084 Assessing the quality of the extracted DNA

A 1% agarose gel was prepared and 5 µl of the DNA preparation was loaded into the gel along with a standard ladder. The gel was stained with 0.5µg/ml ethidium bromide and visualised under UV light.

5.1085 RAPD analysis

The ribosomal gene spacer region was amplified using primers ITS 5 (White *et al.* 1990 in Bulman and Marshall 1997) and ITS 26 (Howlett *et al.* 1992 in Bulman and Marshall 1997). The PCR reactions were carried out in a 25 µl volume containing 10 m*M* Tris-HCl (pH 8.0), 50m*M* KCl, 2.0m*M* $MgCl_2$ 160µM each dNTP, 250µM each primer and 0.6U Taq DNA polymerase (Life Technologies). Amplification reactions were run for 45 cycles with an initial denaturation of 94^0C for two minutes and then 94^0C (30 s), 36^0C (30s) and 72^0C (2 min). A non DNA control was included in all PCR reactions.

5.1086 Gel Electrophoresis

Amplified products together with a 1 kb ladder (Gibco-BRL) were resolved by gel electrophoresis on 1.5%w/v agarose gels with 0.5µgml^{-1} ethidium bromide and electrophoresed in 1 x TBE. Gels were photographed over a UV transilluminator using a Polaroid camera.

5.1087 Restriction digests

One µl of amplified products was transferred to eppendorf tubes. Four µl of the enzymes listed below were transferred to the eppendorf tubes and the mix was then incubated for the length of time and the conditions required for each enzyme. The enzymes used were Rsa1, Cfo, Sma 1, Dde 1, Taq 1, Sau 3A, and Sac 1 (Boehringer-Mannheim New England Biolabs). The restriction digests were run on agarose gels and viewed as above in section 5.186.

5.20 Results

5.201 Measuring data

Four different forms of analysis were used to elucidate the taxonomy of the teleomorph and anamorph relationships of the *Didymella* complex. All analyses were completed in SYSTAT® 7.01 using the morphological measurement data.

5.202 Hierarchical trees

Analysis of the teleomorph state produced five pseudo-taxa (Figure 5.1). The pseudo-taxa were identified as *Didymella exitialis*, *D. graminicola*, *D. loliina*, *D. phleina*, and a group that was unidentified. Published descriptions of type specimens were used to identify and define the different pseudo-taxa.

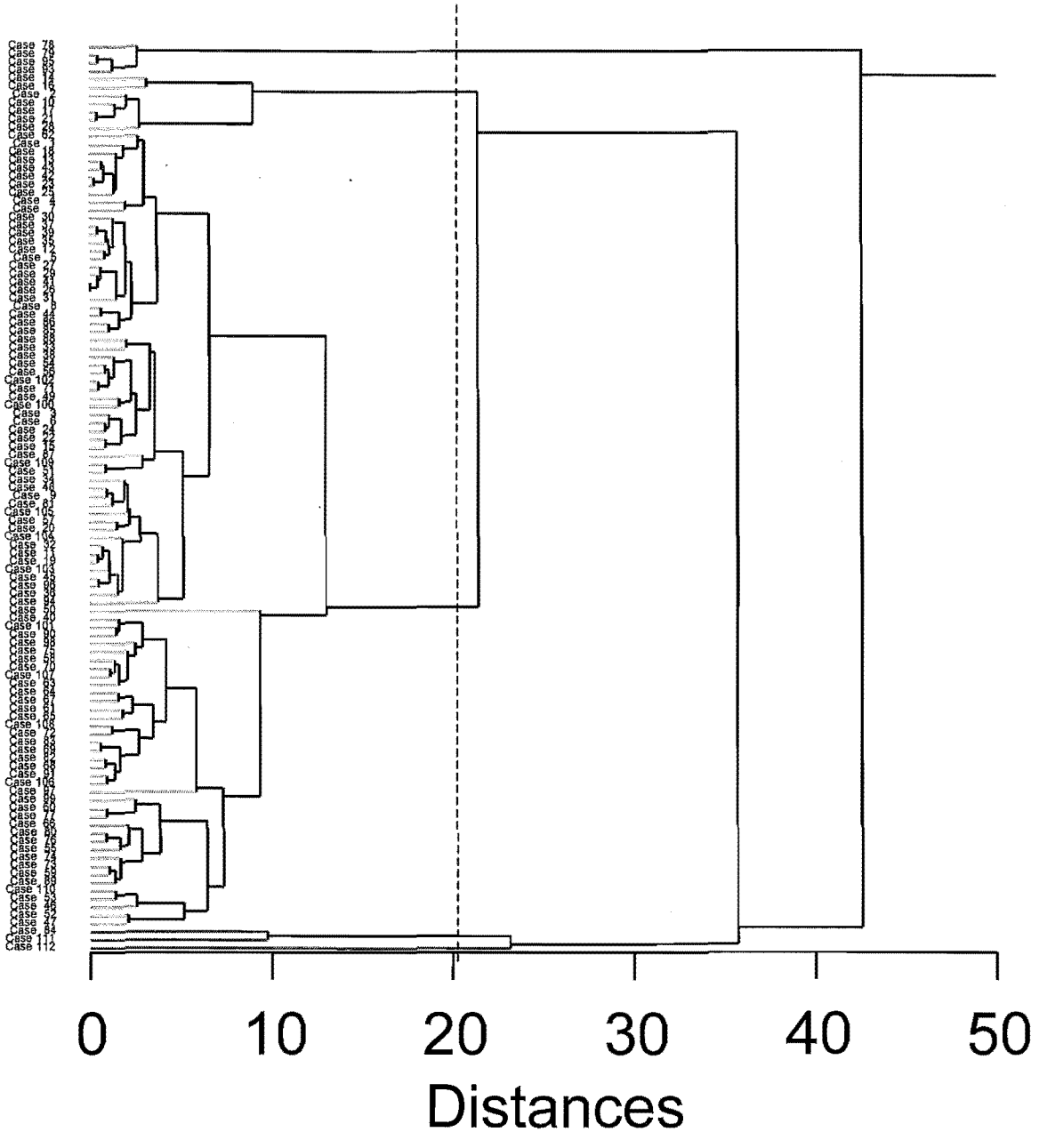

Figure 5.1 Teleomorph hierarchical tree. Pseudo-taxa were calculated at a level of difference of 20μm. Five pseudo-taxa were identified; *Didymella exitialis*, *D. graminicola*, *D. loliina*, *D. phleina*, and a group which was undefined.

Whilst Figure 5.1 uses raw data, a second hierarchical classification using dissimilarity data was produced; this classification exhibited no observable differences. Pseudo-taxa were delineated at a level of 20μm as this was found to be the interquartile range for morphological characters, and produced a number of pseudo-taxa that were neither overly clumped nor overly separated.

The anamorph state produced three pseudo-taxa at the delineation level of 20μm (Figure 5.2). These were identified and defined as being the Ascochyta states of *Didymella exitialis*, *D. Phleina*, and *D. graminicola*. Again, published descriptions of type specimens were used to define pseudo-taxa.

5.203 Additive trees

An additive tree was calculated for the teleomorph and anamorph dissimilarity matrix data. It was considered important to calculate different forms of classifications as often different methods of calculating classifications may give conflicting, and potentially confounding, results. Additive trees use a numerical method that does not necessarily imply that all within-cluster distances are smaller than all between-cluster distances, and that within cluster distances are equal.

The teleomorph additive tree (Figure 5.3) exhibits two clear sections to the classification diagram. The upper division was typified by the published descriptions of type specimens of *D. graminicola* and *D. loliina*. The lower division was typified by the type specimens of *D. exitialis* and *D. phleina*. The presence of the type specimens in these two different divisions suggests that isolates identified in each division are likely to be members of those taxa.

The anamorph additive tree (Figure 5.4) similarly exhibits a division into two groups. However, in this classification the published description of the type specimen of *D. exitialis* is classified as quite separate from the type specimens of *D. graminicola*, *D. loliina*, and *D. phleina*. Of this second grouping, *D. phleina* is still closest to *D. exitialis*.

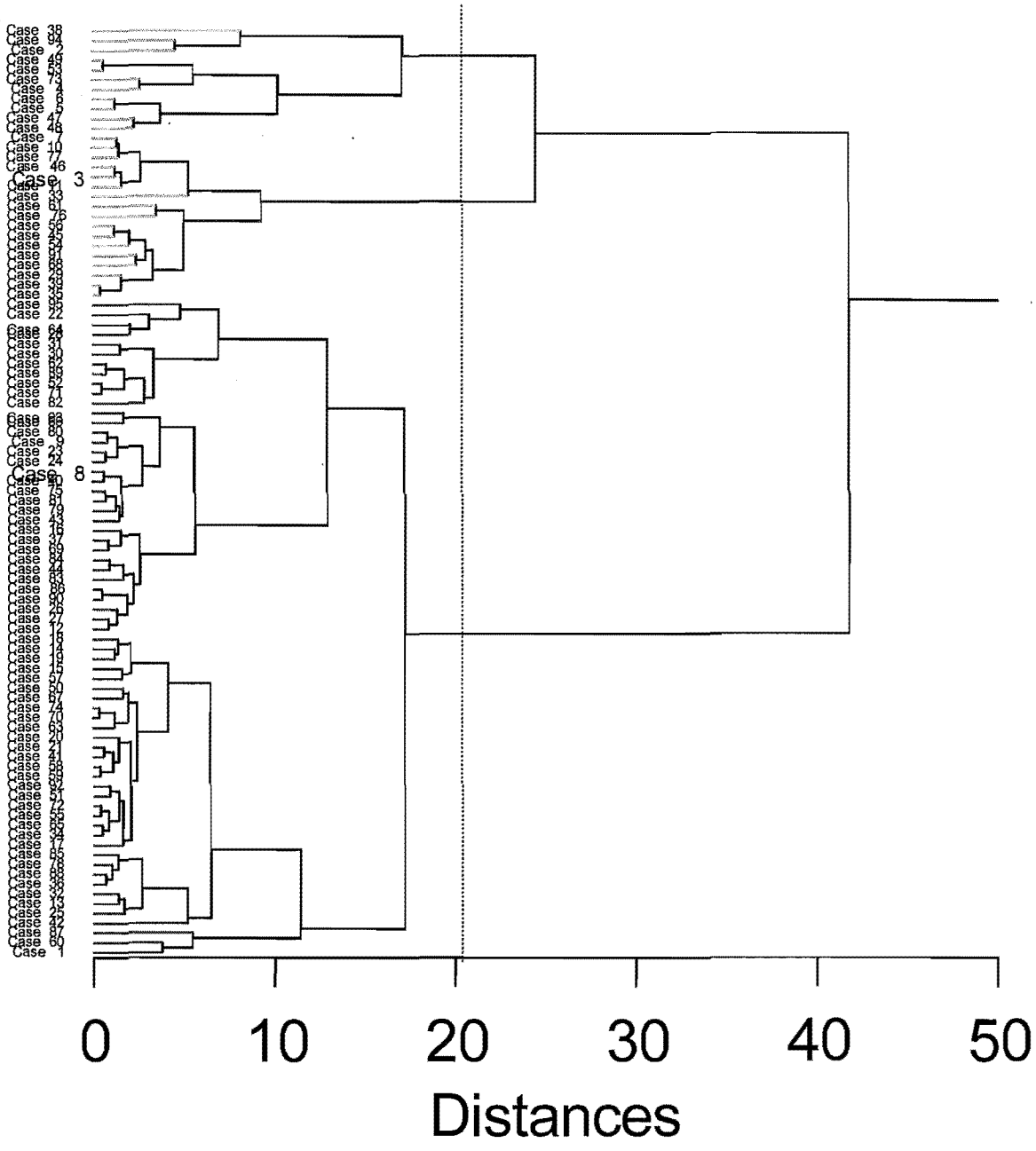

Figure 5.2 Anamorph hierarchical tree. Pseudo-taxa were calculated at a level of difference of 20μm. Three pseudo-taxa were identified which corresponded to the *Ascochyta* states of *Didymella exitialis*, *D. graminicola*, and *D. phleina*.

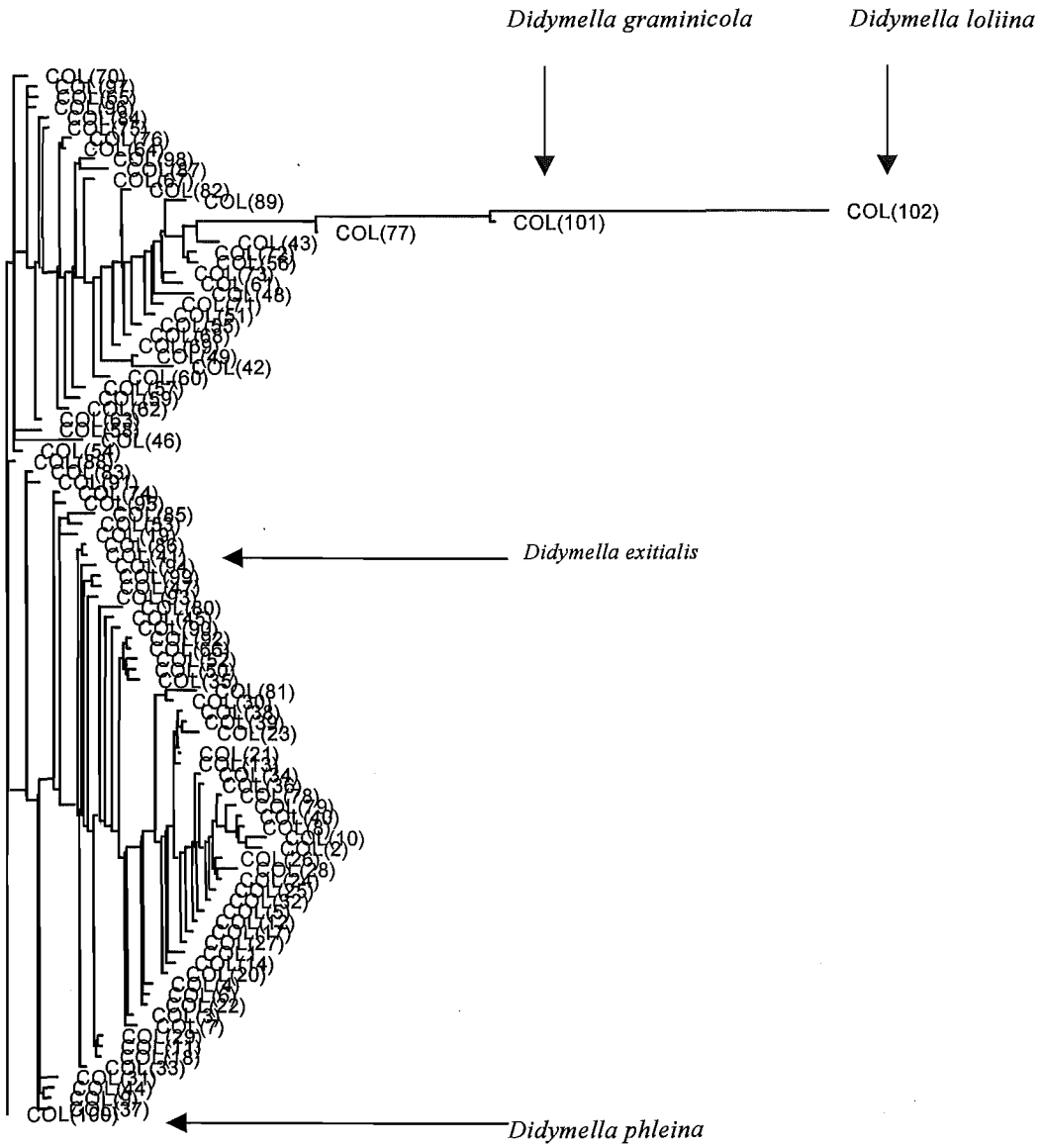

Figure 5.3 Teleomorph state additive tree. Positions of type specimens in the classification are identified. Two main divisions are identified; one containing *D. graminicola* and *D. loliina*, the other *D. exitialis* and *D. phleina*.

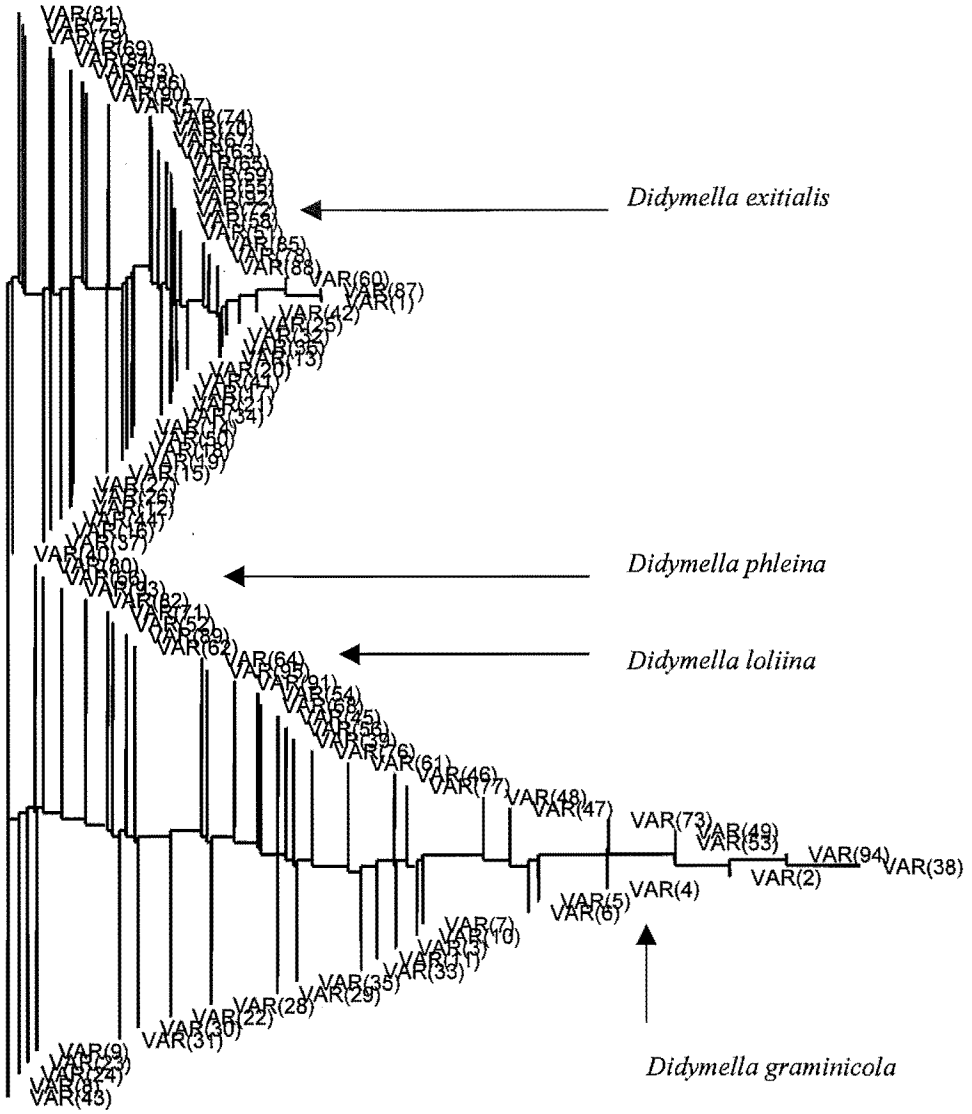

Figure 5.4 Anamorph state additive tree. Positions of type specimens in the classification are identified. Two main divisions are identified; one containing *D. graminicola, D. loliina,* and *D. phleina,* the other *D. exitialis*. This differs from the additive tree.

5.204 Principal components analysis

Principal components analysis of the teleomorph state data produces a relationship as shown in Figure 5.5. Subsequent plotting of morphological characters on the same axes (Figure 5.6) reveals the multidimensional relationship of the isolates. Ascus width is positively correlated with factor 1. This means that isolates with a greater ascus width are located on the right side of the ordination plot. Ascus length is negatively correlated with factor 2 and positively with factor 1; this means that isolates with longer ascus length are located in the bottom right corner of the ordination plot. Pseudothecial diameter is negatively correlated with factor 2, implying that isolates with larger pseudothecial diameters are found on the bottom half of the ordination plot.

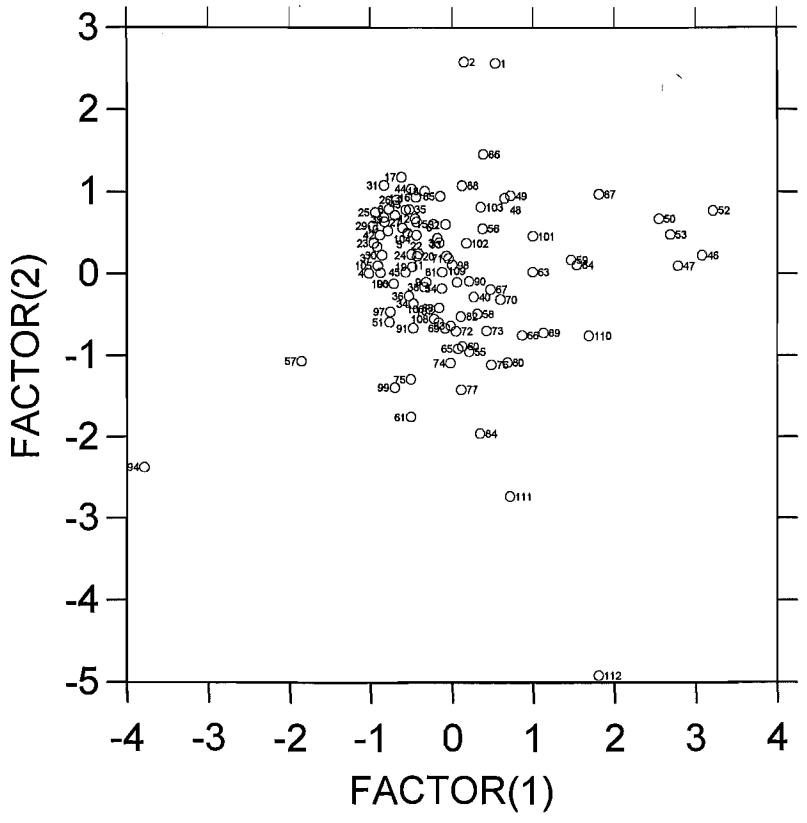

Figure 5.5 Principal components analysis for teleomorph state of Graminicolous *Didymella* spp. Multi-dimensional relationship of isolates reduced to two dimensions. Factor 1 is most strongly correlated with ascus width. Factor 2 is most strongly correlated with pseudothecial diameter (see Figure 5.6).

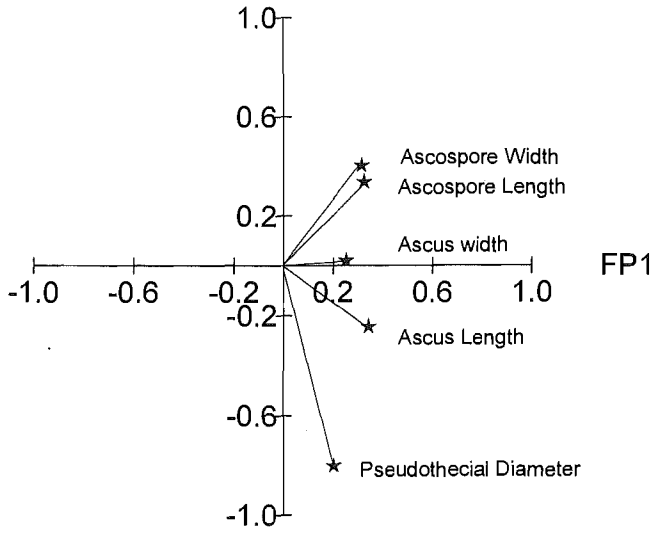

Figure 5.6 Correlation plot of morphological characters on the ordination axes for the teleomorph state. The symbols indicate the direction of positive correlation for each morphological character; the size of the respective lines indicates the strength of the correlation.

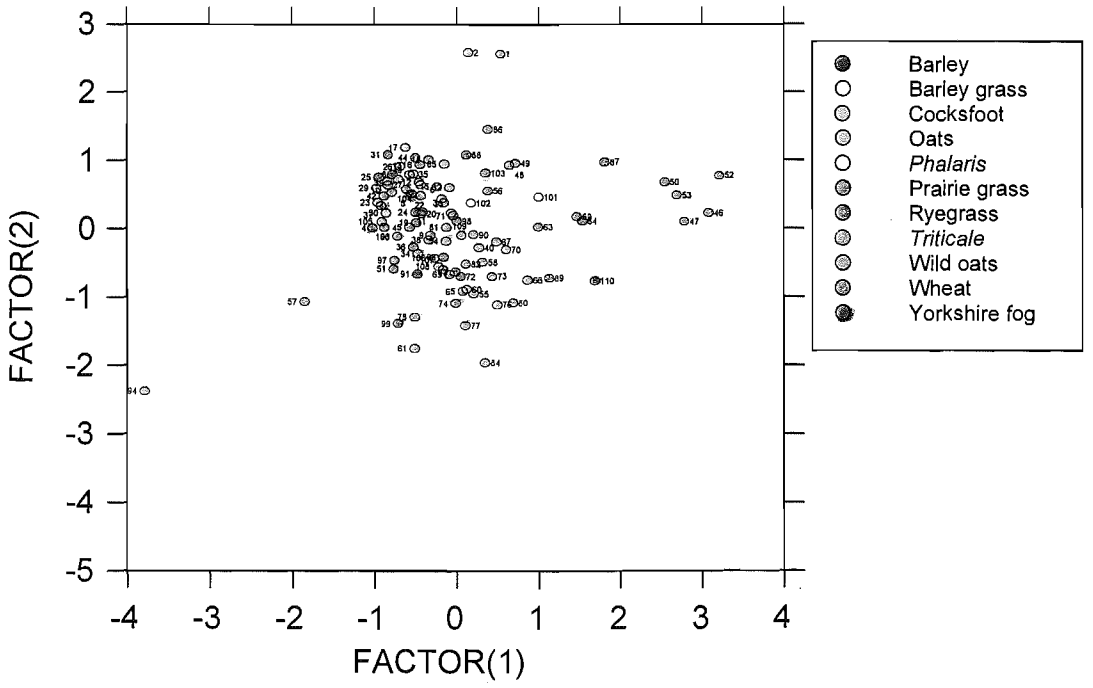

Figure 5.7 Ordination plot of isolates related to host species cultured from for the teleomorph state. Key identifies host species; colour on the ordination plot indicates individual hosts.

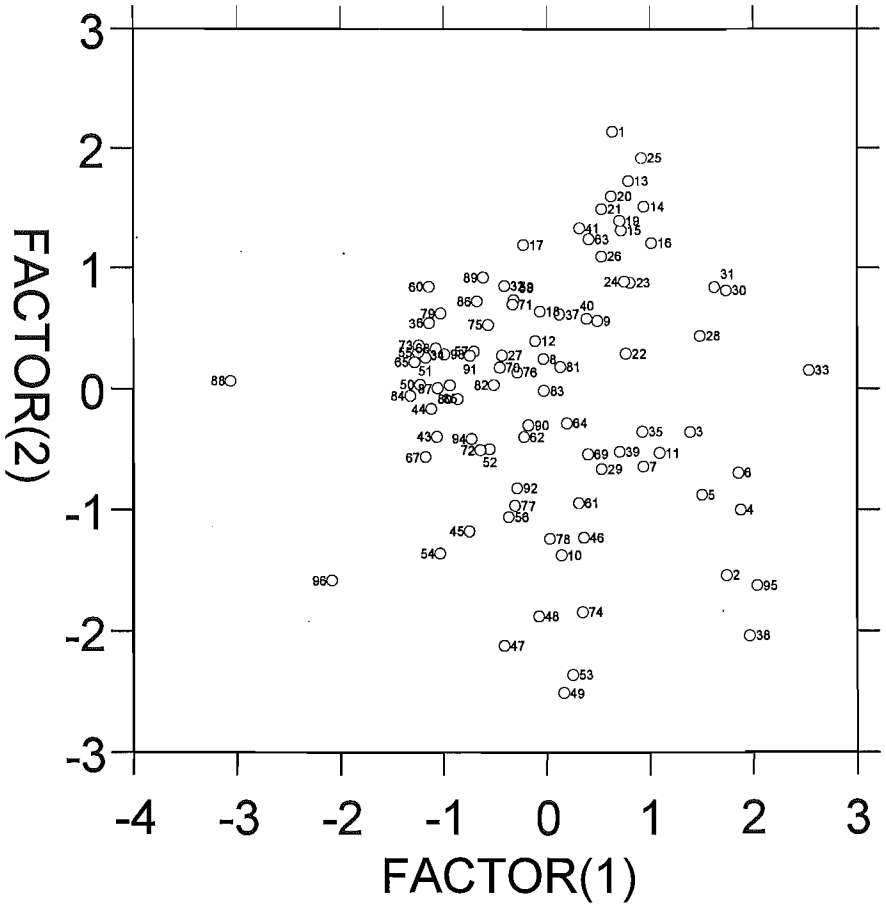

Figure 5.8 Principal components analysis for the anamorph state of Graminicolous *Didymella* spp. Multi-dimensional relationship of isolates reduced to two dimensions. Factor 1 is most strongly correlated with conidial width and length. Factor 2 is most strongly correlated with pycnidial diameter (see Figure 5.9).

The ordination plot of the host species for the teleomorph state of *Didymella* spp. (Figure 5.7) reveals no clear host specificity for teleomorph states. The morphological range suggests that of all the hosts, wheat is likely to have the greatest range of *Didymella* spp.

Principal components analysis of the anamorph state data produces a relationship as shown in Figure 5.8. Subsequent plotting of morphological characters on the same axes (Figure 5.9) reveals the multidimensional relationship of the isolates. Conidial width and length are positively correlated with factor 1 and to a lesser extent factor 2.

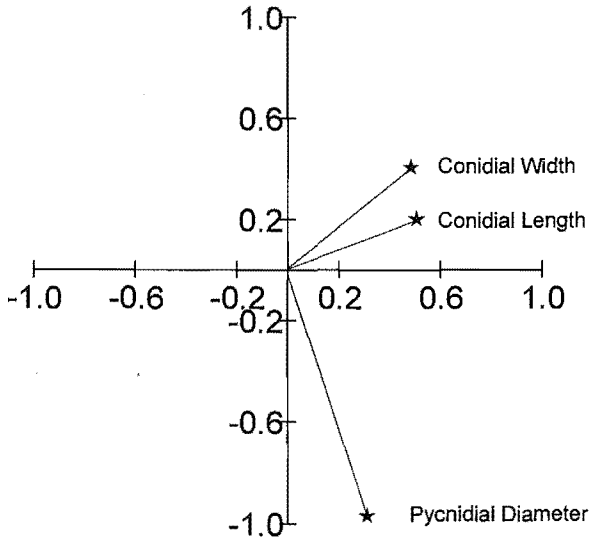

Figure 5.9 Correlation plot of morphological characters on the ordination axes for the anamorph state of Graminicolous *Didymella* spp. The symbols indicate the direction of positive correlation for each morphological character; the size of the respective lines indicates the strength of the correlation.

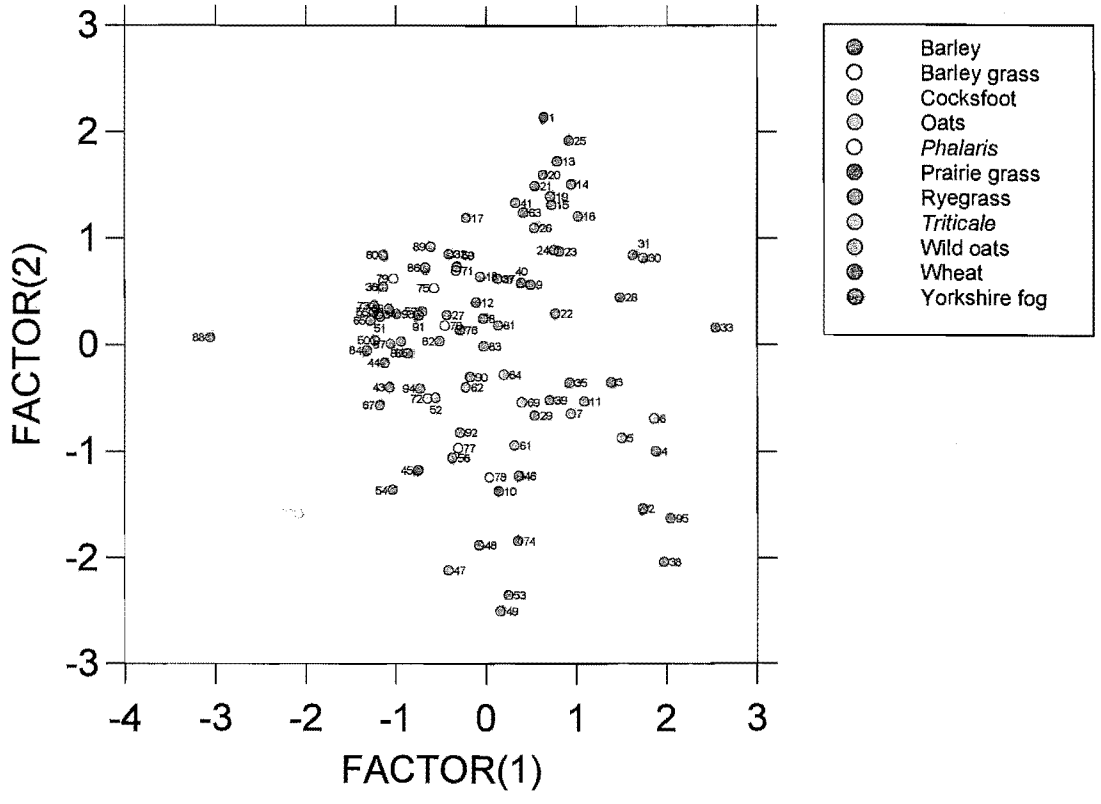

Figure 5.10 Ordination plot of isolates related to host species cultured from for the anamorph state of Graminicolous *Didymella* spp. Key identifies host species; colour on the ordination plot indicates individual hosts.

Pycnidial diameter is strongly negatively correlated with factor 2 of the anamorph ordination plot, and slightly positively correlated with factor 1. This means that isolates located in the lower right quadrant of the ordination plot have larger pycnidial diameters than those located in the upper left quadrant. In terms of conidial width and length isolates in the upper right quadrant possess larger dimensions for these two characters than those isolates located in the lower left quadrant. Interestingly, increasing conidial dimensions and increasing pycnidial dimensions are all positively correlated with factor one.

The ordination plot of the host species for the anamorph state of *Ascochyta* spp. (Figure 5.10) similarly (to the teleomorph), reveals no clear host specificity for anamorph states. However, in the case of the anamorph state, isolates from both wheat and barley not only belong to a wide range of *Ascochyta* species, but also exhibit a wide range of morphological characters.

5.205 Discriminant analysis

Discriminant analysis of the teleomorph state of the *Didymella* spp. was completed using the prior analyses as guides to potential separation. Clear differences were discovered between species (Table 5.3); the greatest difference being 55.855 between *D. exitialis* and *D. phleina.* This means that for the morphological characteristics these two species in the teleomorph state are most distinct. *D. graminicola* and *D. loliina* exhibit the smallest distance between centroid values. The discriminant analysis plot (Figure 5.11) further elucidates the distance relationships of the teleomorph state. The plot was derived using a jack-knifed classification matrix,

Table 5.3 Centroid values for between groups F-matrix elucidating the distance relationships between the teleomorph state of graminicolous *Didymella* spp.

	D. exitialis	*D. phleina*	*D. loliina*	*D. graminicola*
D. exitialis	0.0			
D. phleina	55.855	0.0		
D. loliina	40.020	25.250	0.0	
D. graminicola	23.344	8.687	7.220	0.0

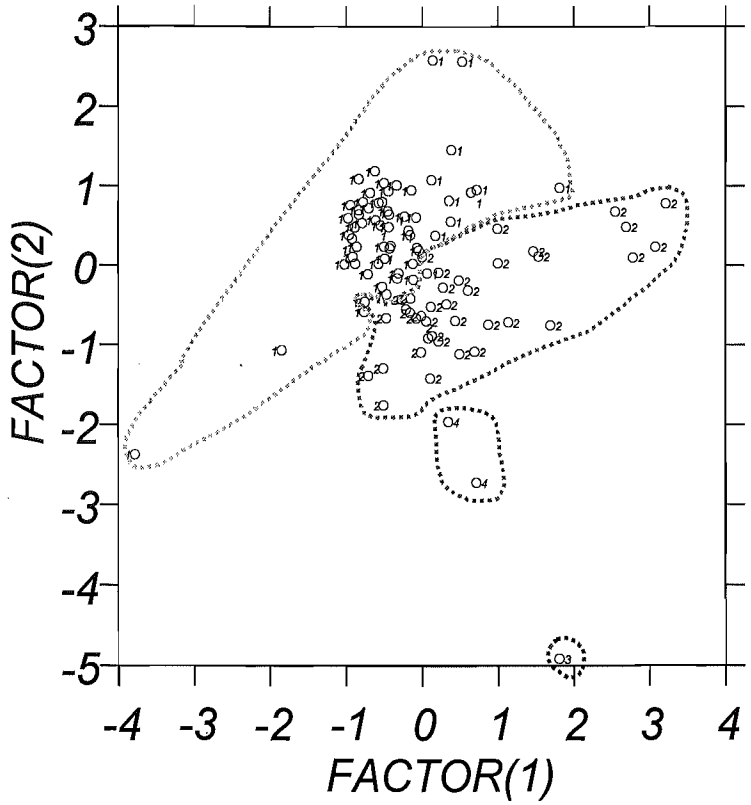

Figure 5.11 Discriminant analysis plot for the teleomorph state of *Didymella* spp. in New Zealand. Groupings indicate the four identified species; (1) *Didymella exitialis*, (2) *D. phleina*, (3) *D. loliina* (Published dimensions), (4) *D. graminicola*.

which reduces the probability of bias when assigning species. This method acts to cross validate data assignations, the inherent problem being that the classification is evaluated using the same cases used to compute it. The relative importance of the different variables in the model was also evaluated with the best predictor being pseudothecial diameter; next ascus length; and finally ascospore width. The canonical scores plot revealed that there was over-lap between the three species but that they could still be distinguished. The species were identified as *Didymella exitialis* (58 isolates), *Didymella phleina* (38 isolates), and one isolate of *Didymella graminicola.*

The anamorph state was also analysed using discriminant analysis. Again, clear differences emerged between different species. The greatest difference was between the *Ascochyta* state of *D. exitialis* and the *Ascochyta* state of *D. graminicola* (Table 5.4). The discriminant analysis plot (Figure 5.12) shows the delimitation of the isolates into four separate groups. A jack-knifed classification matrix identified the species as

Ascochyta state of *D. exitialis* (52 isolates), *Ascochyta phyllachoroides* f. *melicae* (24 isolates), *Ascochyta* state of *D. loliina* (3 isolates), and *Ascochyta* state of *D. graminicola* (14 isolates).

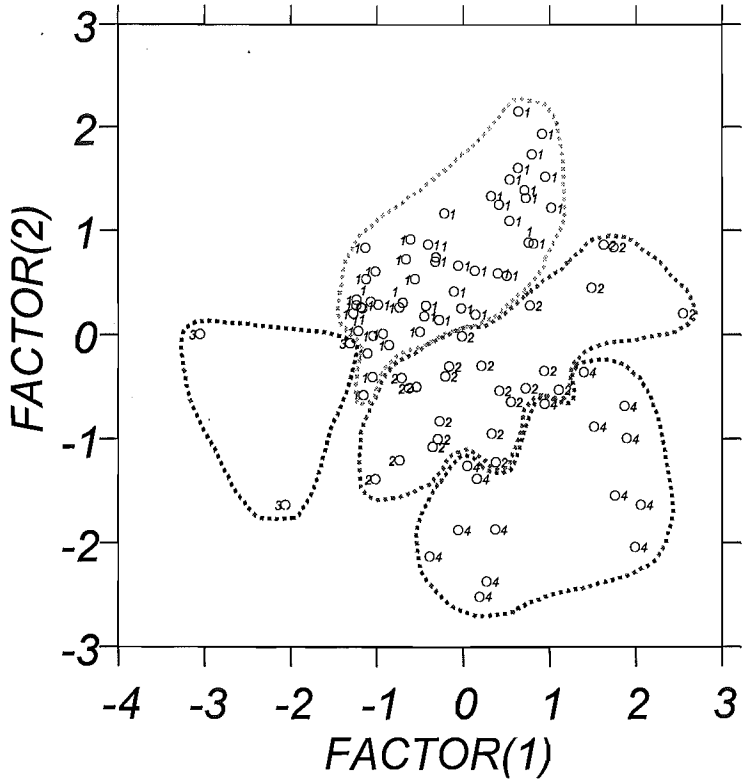

Figure 5.12 Discriminant analysis plot for the anamorph states of *Ascochyta* spp. in New Zealand. Groupings indicate the four identified species; (1) *Ascochyta* state *of Didymella exitialis*, (2) *Ascochyta phyllachoroides* f. *melicae*, (3) *Ascochyta* state *of D. loliina*, (4) *Ascochyta* state *of D. graminicola*.

Table 5.4 Centroid values for between groups F-matrix elucidating the distance relationships for the anamorph state of graminicolous *Didymella* spp.

	Ascochyta state of *D. exitialis*	*Ascochyta phyllachoroides* f. *melicae*	*Ascochyta* state of *D. loliina*	*Ascochyta* state of *D. graminicola*
Ascochyta state of *D. exitialis*	0.0			
Ascochyta phyllachoroides f. *melicae*	52.433	0.0		
Ascochyta state of *D. loliina*	13.166	17.877	0.0	
Ascochyta state of *D. graminicola*	160.361	35.406	35.641	0.0

Table 5.5 Mean range and range for morphological characters for the teleomorph states of *Didymella exitialis*, *D. phleina*, and *D. graminicola*. Mean range is the arithmetic mean ± 1 standard deviation. The second range in each column is the range.

Species	Pseudothecia diameter (µm)	Ostiole Diameter (µm)	Ascus length (µm)	Ascus width (µm)	Ascospore length (µm)	Ascospore width (µm)
D. exitialis	92-111	20-26	40-47	8.5-10.7	13.5-16.5	3.4-4.6
	84.2-119		37.4-50.4	7.9-13.6	12.3-18	2.7-5.9
D. phleina	120.4-137		44-55	8.9-10.8	14.3-17.9	3.7-4.9
	114.8-143.4		45.29-64	8.2-12.3	13.1-21.8	4-5.5
D. graminicola	158-180		45-47.1	9.9-11	15.2-16.5	3.5-4
	153.9-184.5		44.6-47.5	9.7-11.2	14.9-16.8	3.4-4.1

Table 5.6 Mean range and range for morphological characters for the anamorph of the *Ascochyta* state of *Didymella exitialis*, *D. loliina*, *D. graminicola*, and *Ascochyta phyllachoroides* f. *melicae*. Mean range is the arithmetic mean ± 1 standard deviation. The second range in the column is the range.

Species	Pycnidial diameter (µm)	Conidial length (µm)	Conidial width (µm)
Ascochyta* state of *D. exitialis	114.9-140.7	14.9-18	3.6-5.4
	104-147.4	15-19.2	4-6
Ascochyta phyllachoroides* f. *melicae	158.9-185.6	15.2-19.8	3.9-5.5
	150-193.6	13.9-24.3	3.3-6
Ascochyta* state of *D. graminicola	202.1-239.8	15.9-19.6	3.9-6.2
	196.2-242.2	15.3-19.6	3.3-6
Ascochyta* state of *D. loliina	99.1-169.7	8-12.6	3.3-4.1
	99-170	8.2-12.7	3.1-4.1

5.206 *D. exitialis* and the *Ascochyta* state of *D. exitialis*

Generally, the range of pseudothecial diameter, ascus length and width, and ascospore width were similar to those dimensions published by Punithalingam (1979b) (Tables 5.5 and 5.6). The ascospore length was greater (12.3-18µm) than that published by Punithalingam (1979b)[12-14µm]. The diameter of the pycnidia of the *Ascochyta* state of *D. exitialis* were found to be larger (114.9-140.7µm) than the diameter of ≤ 120µm published by Punithalingam (1979b). The measurements of *D. exitialis* and its anamorph obtained from different hosts are all similar, although there is variability between the isolates present on different hosts but no more than there is within an

individual isolate (Tables 5.7 and 5.8). *D. exitialis* has not been recorded on *Agropyron repens* and *Lolium perenne* before.

Table 5.7 Mean ranges (mean ± sd) of perithecial diameter, ostiole diameter, ascus length and width and ascospore length and width of *Didymella exitialis* on graminicolous hosts. Where only one measurement is given for each of the characters is due to the fact only one isolate of *D. exitialis* could be obtained from the host species in question and the measurements are mean values only. All measurements are in µm.

Host	Perithecial diameter	Ostiole diameter	Ascus length	Ascus width	Ascospore length	Ascospore width
Wheat	92.9-115.6	19.8-26.9	40-47.4	9.2-10.4	13.8-16.2	3.5-4.5
Barley	87.7-106.6	19-25.9	39.2-47.1	9.1-11.2	13.7-16	3.8-4.4
Cocksfoot	111-113		39.4-48.5	6.6-9.87	13.9-15	2.2-4.56
Ryegrass	106.6		44.8	8.2	13.6	4.3
Oats	82.8-101	19.8	41.9-45.4	9.9	14-16	4
Triticale	88.5	20.7	41.9	7.9	16.4	5.9
Barley grass	105.1		48.2	9.9	16.6	4
A. repens	108.5		48.9	9.9	19.4	4
Yorkshire fog	110-111		40.3-42.1	7.2-12	14.2-16	3.7-5.2
Wild oat	114.6		43.2	7.1	13.1	4.9

Table 5.8 Mean ranges (mean ± sd) of pycnidial diameter and conidial length and width of the *Ascochyta* state of *Didymella exitialis* on graminicolous hosts. Where only one measurement is given for each of the characters is due to the fact only one isolate of *D. exitialis* could be obtained from host species in question and the measurements are mean values only. All measurements are in µm.

Host spp.	Pycnidia diameter	Conidial length	Conidial width
Wheat	114.6-141.2	15.5-18.3	3.6-5.7
Barley	112-135	15-19	3.9-6
Phalaris	117-150.5	13.8-18.1	4-4.2
Barley grass	119.3-144	16.5-17.4	4.0
Wild oat	115.3-136.4	14.7-15.4	3.7-4.6
Yorkshire fog	111.3-140.6	13.9-16.6	4.1
Prairie grass	116.8-137.9	14-17.2	3.4-4.7
Cocksfoot	141	16.2	4.0
Triticale	133.6	16.8	4
Ryegrass	102.6	13.9	4

5.207 *D. phleina* and *Ascochyta phyllacoroides* f. *melicae*

The mean range of *D. phleina* tended to be smaller than the measurements published by Punithalingam (1979b), but generally similar (Tables 5.5 and 5.6). The characters measured were approximately the same as those published by Punithalingam for the *Ascochyta phyllacoroides* f. *melicae*. The measurements of the isolates obtained from the different hosts were all within the range of the published descriptions (Tables 5.9 and 5.10). Importantly, *D. phleina* had previously not been recorded on wheat.

Table 5.9 Mean ranges (mean ± sd) of perithecial diameter, ostiole diameter, ascus length and width and ascospore length and width of *Didymella phleina* on graminicolous hosts. Where only one measurement is given for each of the characters, only one isolate of *D. phleina* could be obtained from host species in question and the measurements are mean values only. All measurements are in µm.

Host	Perithecial diameter	Ostiole diameter	Ascus length	Ascus width	Ascospore length	Ascospore width
Wheat	115.7-139	26.5-34.7	44.5-55.76	8.7-10.83	14.2-18.2	3.8-5.1
Barley	116.8-134		44.3-49.5	8.8-12	14.6-17.3	2
Prairie grass	118-132.6		35.9-47.8	8.3-9	15.4-16.5	3.7-4.6
Barley grass	111.8-144		48.7-58.9	9.8-11.3	17.9-22.5	4-5.8
Ryegrass	141		41	9.2	14.1	3.8

Table 5.10 Mean ranges (mean ± sd) of pycnidial diameter and conidial length and width of the *A. phyllachoroides* f. *melicae* on graminicolous hosts. Where only one measurement is given for each of the characters, only one isolate of *A. phyllachoroides* f. *melicae* could be obtained from host species in question and the measurements are mean values only. All measurements are in µm.

Host	Pycnidial diameter	Conidial length	Conidial width
Wheat	162.7-188.4	16.2-20.5	4.3-5.8
Barley	165-189	15-18	4.2-5.1
Phalaris	184.6	13.9	4.7
Barley grass	157.1	14.1	4
Wild oat	158.8-189	15.7-16.6	4-4.2
Prairie grass	157-178.4	15.4-17.7	3.8-5
Cocksfoot	153.3	16.8	4.5

5.208 *D. graminicola* and the *Ascochyta* state of *D. graminicola*

The mean ranges for all the characters measured were lower than the published dimensions (Table 5.5). The anamorph characters measured were all larger than published dimensions (Punithalingam 1979b) (Table 5.6). *D. graminicola* had previously not been recorded on any of the hosts apart from ryegrass (Tables 5.12 and 5.13).

5.209 *D. loliina* and the *Ascochyta* state of *D. loliina*

The dimensions of the characters measured were approximately the same as those published by Punithalingam (1979b) for the *Ascochyta* state. *D. loliina* has not been recorded on Yorkshire fog previously.

Table 5.11 Mean ranges (mean ± sd) of perithecial diameter, ostiole diameter, ascus length and width and ascospore length and width of *Didymella graminicola* on graminicolous hosts. Where only one measurement is given for each of the characters, only one isolate of *D. graminicola* could be obtained from host species in question and the measurements are mean values only. All measurements are in µm.

Host	Perithecial diameter	Ostiole diameter	Ascus length	Ascus width	Ascospore length	Ascospore width
Wheat	158.4		47.1	9.9	15.2	4
Ryegrass	220		55	12.5	13	4.3

Table 5.12 Mean ranges (mean ± sd) of pycnidial diameter and conidial length and width of the *Ascochyta* state of *D. graminicola* on graminicolous hosts. Where only one measurement is given for each of the characters, only one isolate of *Ascochyta* state of *D. graminicola* could be obtained from host species in question and the measurements are mean values only. All measurements are in µm.

Host spp.	Pycnidial diameter	Conidial length	Conidial width
Wheat	208-245.6	16.3-18.8	3.2-5.5
Barley	198-220	17-22	5.7-6.8
Phalaris	197.7	15.3	4.6
Wild Oat	99.3	8.2	3.3
Triticale	199.9	16.1	5.9

Table 5.13 Mean ranges (mean ± sd) of pycnidial diameter and conidial length and width of the *Ascochyta* state of *D. loliina* on graminicolous hosts. Where only one measurement is given for each of the characters, only one isolate of the *Ascochyta* state of *D. loliina* could be obtained from host species in question and the measurements are mean values only. All measurements are in µm.

Host spp	Pycnidial diameter	Conidial length	Conidial width
Yorkshire fog	121-209	11.6-19.4	3.7-6.3

5.210 Culture descriptions

All cultures of *Didymella* spp. grown on PDA were floccose or tufted. The colouration of cultures can be separated into 4 main groups which do not correspond to the species descriptions. These groups are:

1/ grey olivaceous top and the reverse is brick and greenish yellow.

2/ grey olivaceous top with buff or white blotches and the reverse is brick and straw yellow.
3/ grey olivaceous top with buff or white blotches and the reverse is brick, straw yellow and greenish yellow.
4/ buff white and grey olivaceous top and buff white reverse with grey olivaceous and brick red at the point of inoculation.

There does not appear to be any host specificity of isolates with regard to their culture colouration. Cultures of different colours can be obtained from a subcultured single ascospore isolate grown under identical conditions or isolates from the same leaf piece (Plates 5.1-5.12). There are also no differences between the species as to the colouration of the culture, and a considerable amount of variation exists within isolates of *D. exitialis* and *D. phleina*. The cultures of *D. graminicola* have the same level of variation of colony colour within the isolates.

5.211 Shape of conidia and ascospores, and their colouration

Colouration of conidia was variable; some isolates initially produced hyaline conidia and then straw yellow conidia as the culture aged. No colouration of ascospores was observed throughout the course of this study. Ascospore and conidial shape was also extremely variable. For example a culture identified as *D. exitialis* at least half the conidia were found to fit the shape descriptions published for *D. phleina* (data not shown). Ascospore shape was also subject to this variability.

5.212 Molecular analyses

5.2121 CHEF and RAPD

Optimisation of the amount of mycelium required for a CHEF analysis was not achieved in this study. Chromosomal DNA was found to have degraded during plug preparation. Further CHEF analyses investigated treating the plug with RNase A, but this did not solve the degradation problem.

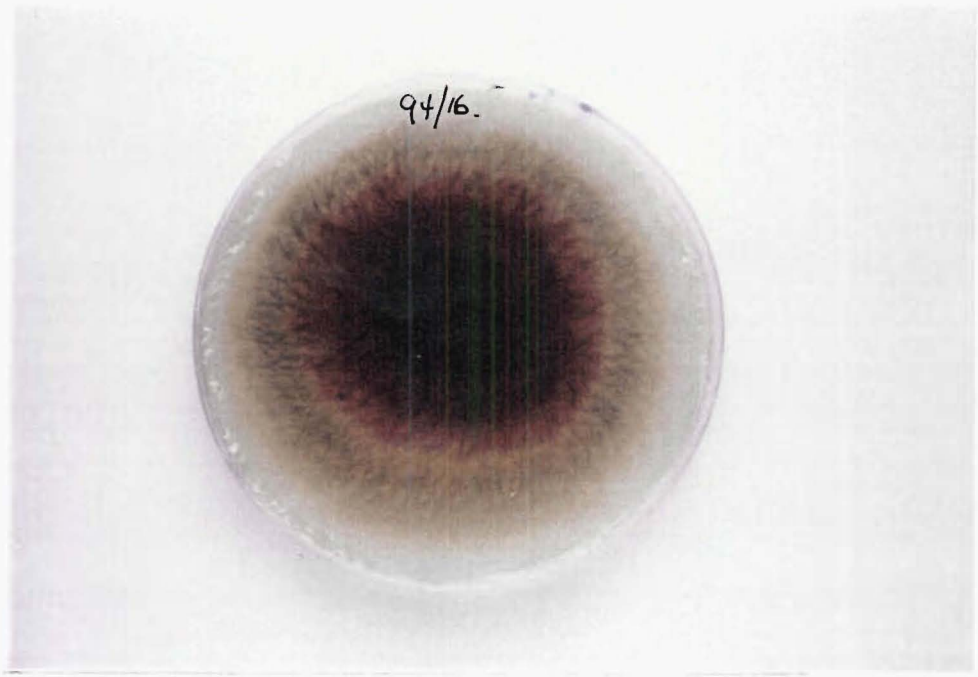

Plate 5.1 The reverse of an isolate of *D. exitialis* (94/16 isolated from cocksfoot) Brick red with a green olivaceous outer concentric ring.

Plate 5.2 The top of 94/16 with floccose green olivaceous aerial mycelium

Plate 5.3 Reverse of an isolate of *D. exitialis* (94/4 isolated from wheat) with green olivaceous at the point of inoculation and a small area of brick red with a buff background.

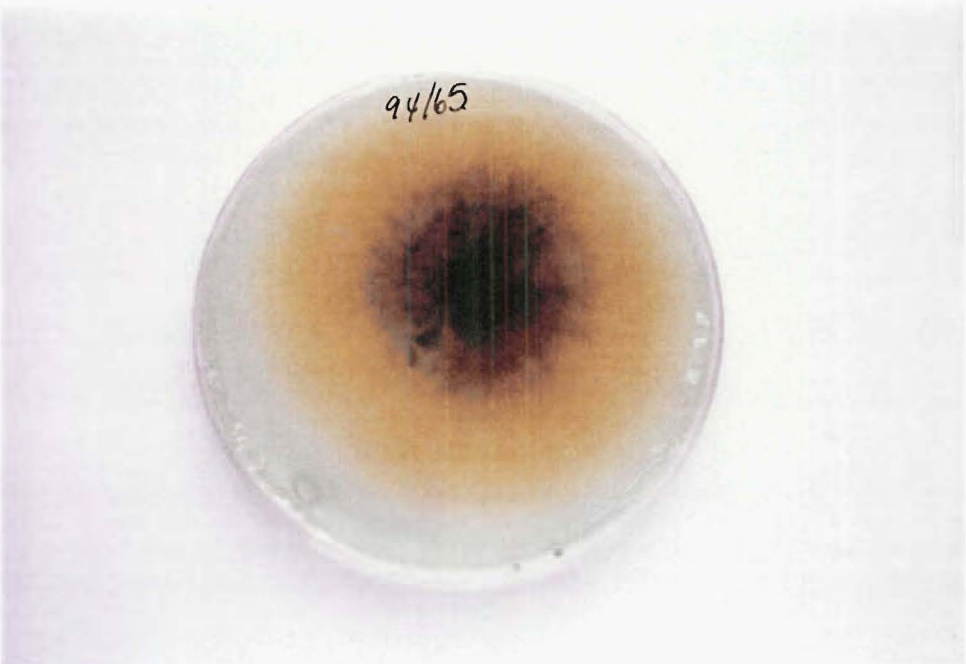

Plate 5.4 The reverse of a culture of *D. exitialis* (94/65 isolated from Prairie grass) with brick red and a background of straw yellow.

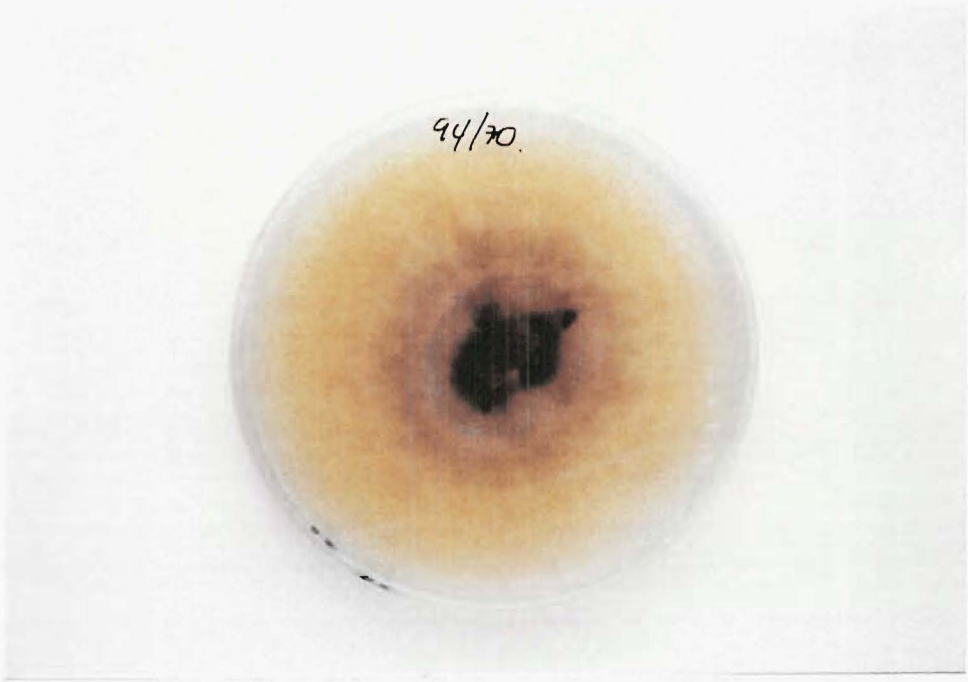

Plate 5.5 The reverse of a culture of *D. exitialis* (94/70 isolated from Barley grass) with brick red at the point of inoculation and two concentric rings of brick red and a straw yellow background

Plate 5.6 The top of a culture of *D. exitialis* (94/70) which is grey olivaceous with white blotches. All the other isolates of *D. exitialis* apart from 94/16 plated had grey olivaceous tops with white or buff blotches.

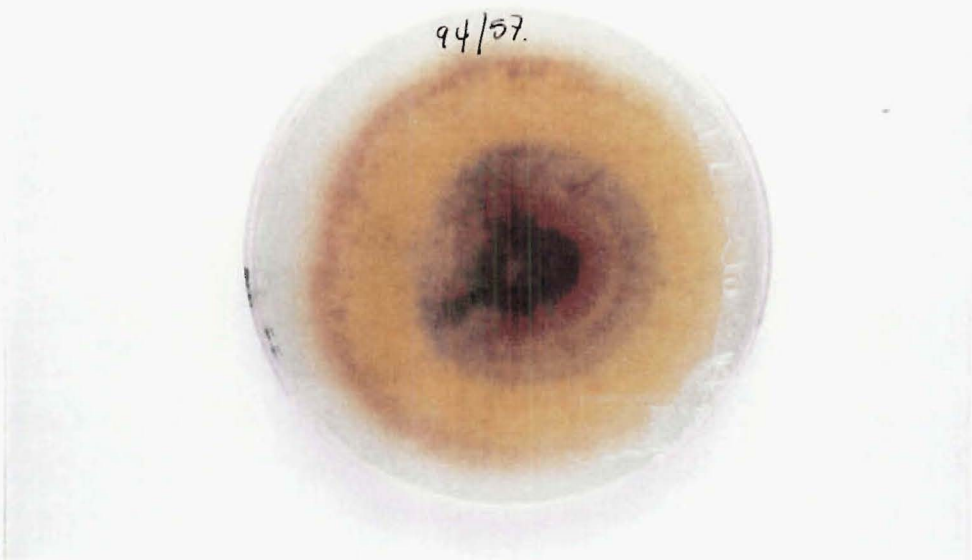

Plate 5.7 The reverse of a culture of *D. phleina* (94/57 isolated from barley)which has brick red at the point of inoculation and then a concentric ring of green olivaceous, a straw yellow background and at the actively growing edge of the culture a concentric ring of brick red.

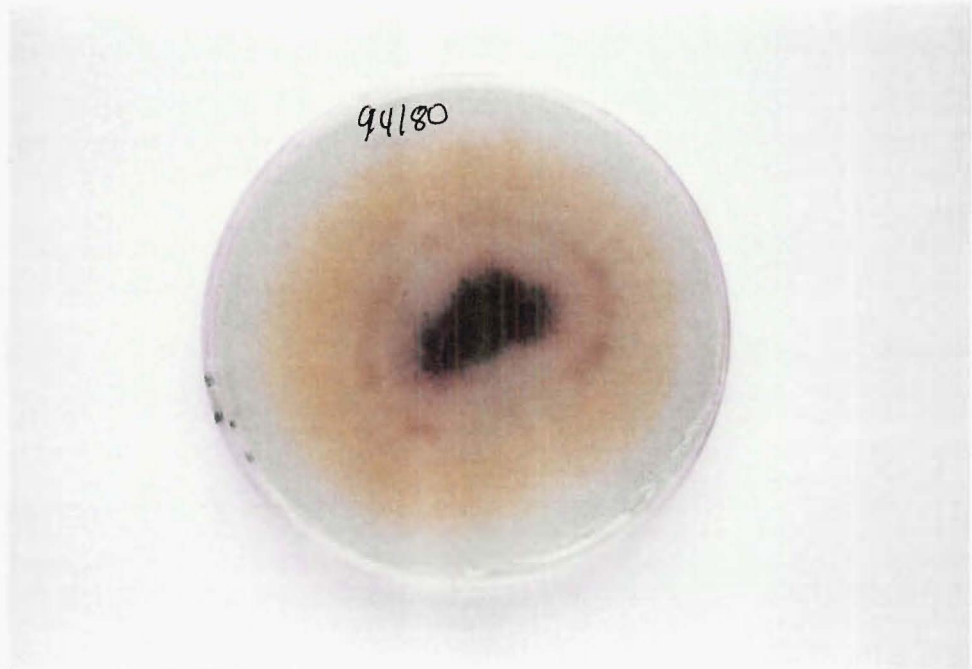

Plate 5.8 The reverse of a culture of *D. phleina* (94/80 isolated from wild oat) with brick red at the point of inoculation and one concentric ring of brick red with a straw yellow background.

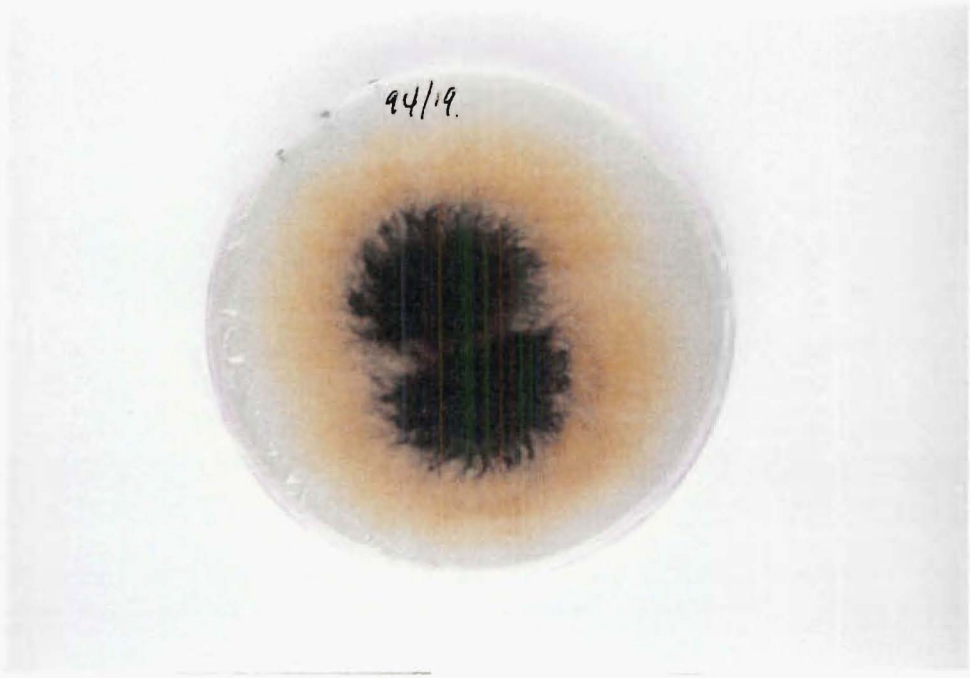

Plate 5.9 The reverse of a culture of *D. phleina* (94/19 isolated from barley) with green olivaceous at the point of inoculation and a straw yellow background.

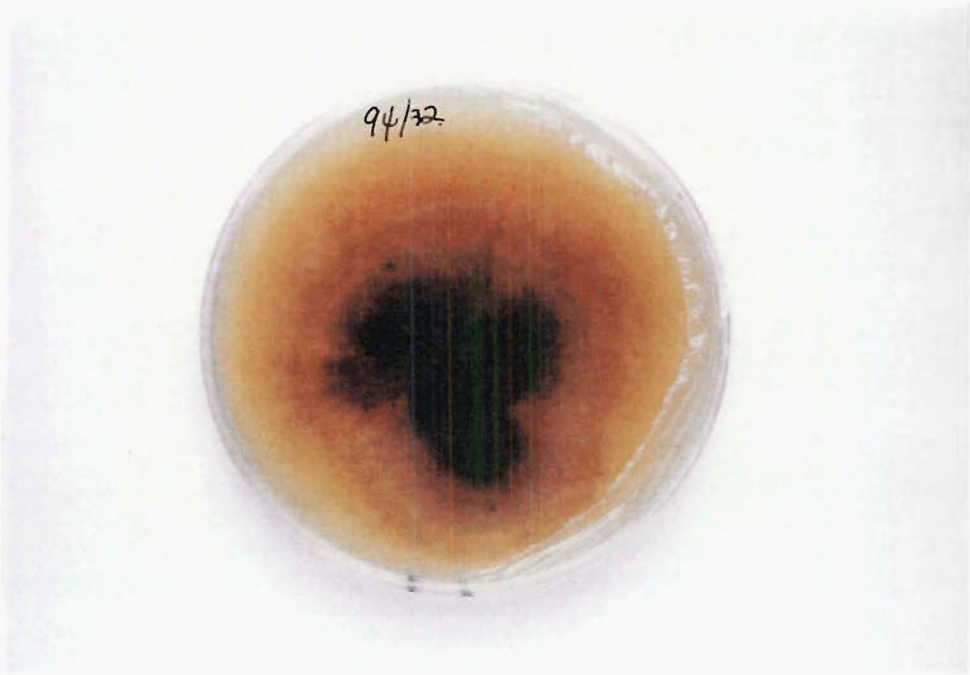

Plate 5.10 The reverse of a culture of *D. phleina* (94/72 isolated from barley) with brick red at the point of inoculation and two concentric ring of brick red and a straw yellow background

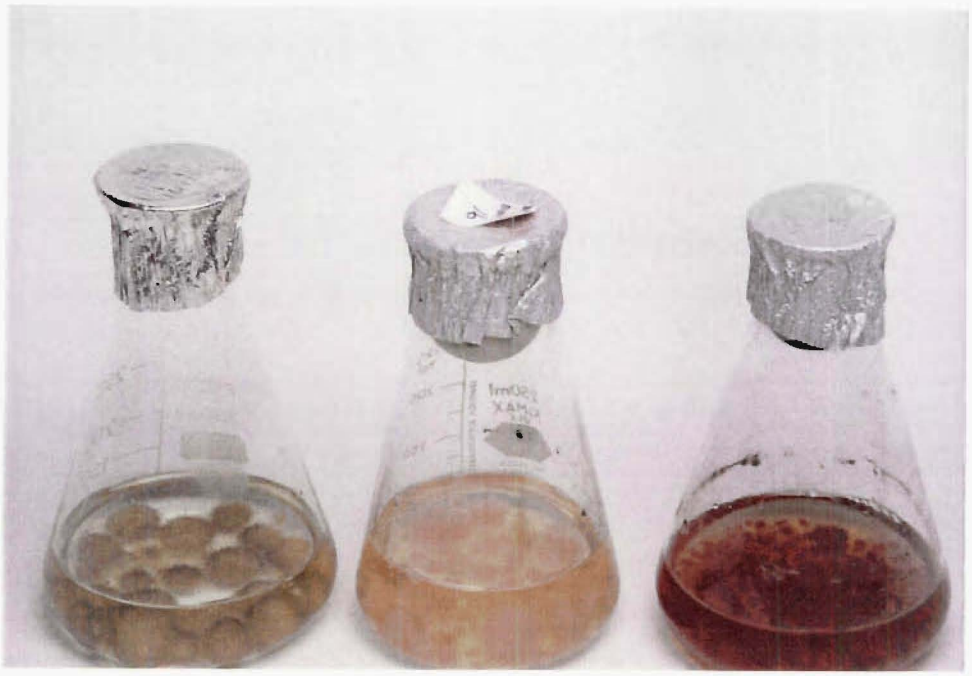

Plate 5.11 The differences in colouration of one single ascospore isolate cultured in PDB all flasks received the same treatment.

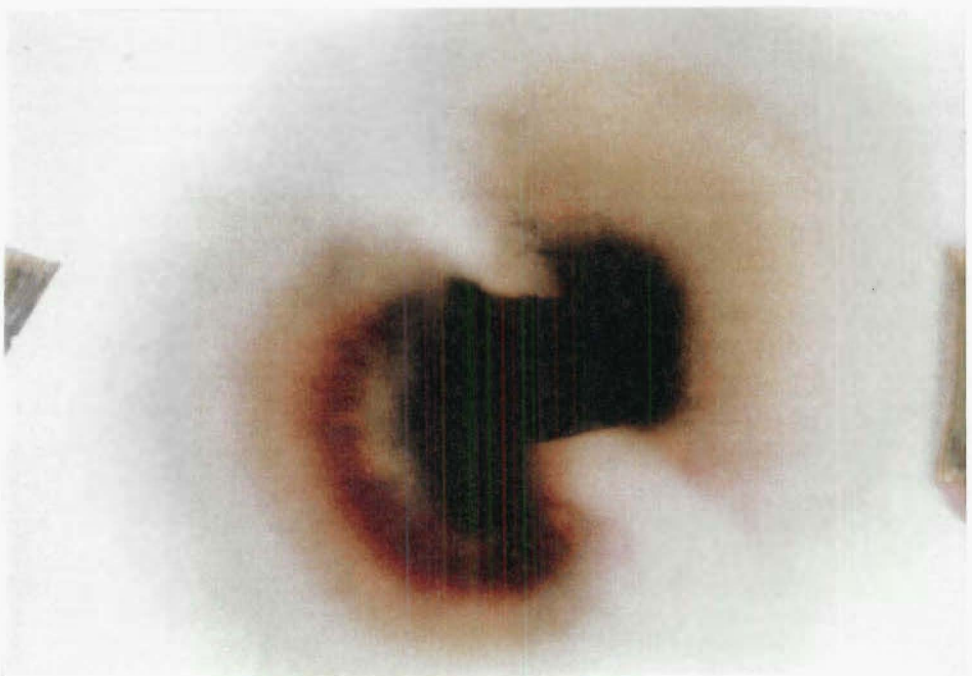

Plate 5.12 Colouration of two isolates growing from one leaf piece. Note the differences in colour. These two isolates had very similar measurements and were classified as *D. exitialis*.

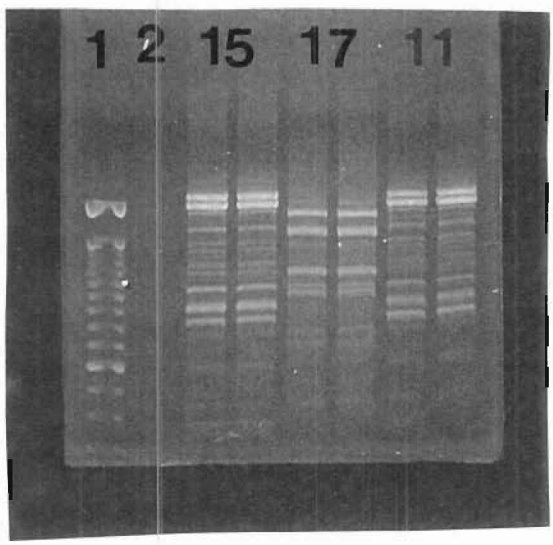

Plate 5.13 Agarose gel exhibiting the RAPD products of 3 graminicolous *Didymella* isolates 11, 15, and 17. The banding pattern for 17 is different to the other two isolates. In lane 1 is the 1kb ladder; lane 2 is a negative control

Plate 5.14 Agarose gel showing the restriction digests (enzymes Rsa1, cfo1) of five Graminicolous *Didymella* isolates (1, 11, 15, 17, and 23). Lane 1, 1kb ladder; Lane 2, 15 Rsa1; Lane 3, 15 Cfo1; Lanes 4 and 6, 17 Rsa1; Lanes 5 and 7, 17 Cfo1; Lane 8, 1 Rsa1; Lane 9, 1Cfo1; Lane 10, 23 Rsa1; Lane 11, 23 Cfo1; Lane 12, 11 Rsa1; Lane 13 11 Cfo1.

5.2122 RAPD

Isolate 96/17 was found to have a different banding pattern from the other isolates, which is confirmed by the morphological analysis. Isolate 96/17 was identified as *D. phleina* and the other isolates (95/11 and 95/15) were identified as *D. exitialis* (Plate 5.13).

5.2123 Restriction digests

Only one enzyme allowed distinction between isolates, the other enzymes used did not provide a method of suitably distinguishing between the isolates as the products for all the RAPD products had identical bands for each of the individual enzymes used. The one enzyme that did allow distinction was Rsa1. Isolate 1 and isolate 15 exhibited three bands whereas all other isolates exhibited only two bands. (Plate 5.14)

5.30 Discussion

5.301 *Didymella* species identified on Graminicolous hosts in New Zealand.

Cluster and factor analyses of selected morphological characters in several isolates of graminicolous *Didymella* spp. have shown that there are consistent and adequate morphological differences to distinguish at least three groups for the teleomorph state, corresponding to *D. exitialis*, *D. phleina* and *D. graminicola*. For the anamorph state, consistent differences were obtained to distinguish at least four and possibly five groups, these being *Ascochyta* states of *D. exitialis*, *Ascochyta phyllachoroides* f. *melicae*, *Ascochyta* state of *D. graminicola* and *D. loliina*. Discriminant analysis allowed the identification of linear combinations of morphological characters which best separated the characters that contributed most to group separation. Perithecial diameter was the most important character for group separation followed by ascospore length and width. Pycnidial diameter was the most important character for distinguishing between species in the anamorph state.

5.302 Teleomorph

Three species were identified from the 145 isolates of *Didymella* species isolated from graminicolous hosts. The three species were *D. exitialis*, *D. phleina* and *D. graminicola*. The measurements obtained from the current study are similar in most instances to those published by Punithalingam (1969, 1979b). The shape of the ascospores was variable and could not be used to distinguish between the different species. No examples of colouration of ascospores could be seen in any of the isolates examined, regardless of species. This differs from Allitt (1986) who found that late colouration of the ascospores of *D. exitialis* occurred.

5.303 Anamorph

Four, possibly five species were identified from the 136 isolates of the anamorph state obtained from graminicolous hosts. These species included the *Ascochyta* state of *D. exitialis* and the *Ascochyta* state of *D. graminicola.* Two isolates (isolates 3 and 7 in appendix 4) identified as the *Ascochyta* state of *D. graminicola* could also be identified as *A. sorghi*. Examples of the teleomorph of these isolates existed and these were also identified as *D. graminicola*. This is an area where molecular work would be useful to confirm the observations of this study.

The large number of isolates measured during the course of this study (129 for the teleomorph and 132 for the anamorph) allowed a thorough sampling of population variation of each of the species. The differences obtained between the data of Punithalingam (1979b) and those in the present study can be explained in most instances. Punithalingam (1979b) measured type specimens which, although they were selected at random from the living population, cannot be representative of the species in question, and hence can only be related at the time of collection (Talbot 1971).

Didymosphaeria loliina

One isolate was identified as the *Ascochyta state of D. loliina.* The host was Yorkshire fog. This fungus species had previously only been known from ryegrass seed, of which only one isolate was obtained (Punithalingam 1979b). To confirm the presence

of this species more isolates of *D. loliina* would required so as measurements were not based on only a few examples.

Didymella graminicola

Previously, *D. graminicola* had only been isolated from *Lolium perenne* and *L. multiflorum* seeds by U.G. Schlösser (Punithalingam 1979b). Currently New Zealand is experiencing difficulty in supplying the United States of America with ryegrass seed as the Americans claim that New Zealand ryegrass seed is infected with *D. graminicola* and the infection is seedborne (Grbavac pers. comm. 1996). The teleomorph was obtained on ryegrass and wheat showing that it does exist in New Zealand. It is not known if *D. graminicola* is seedborne, but in Chapter two *D. exitialis* and *D. phleina* were found not to be seedborne. More research is required to establish whether *D. graminicola* is seedborne. The anamorph state is also present in New Zealand but no isolates were obtained from ryegrass, as the teleomorph could not be isolated to pure culture. Isolates were obtained from barley, phalaris, wild oat and triticale. *D. graminicola* has previously never been recorded occurring on the above hosts.

The current study has not been able to clarify the confusion that exists as to identity of the anamorph state. Three species *A. graminicola* Sacc., *A. graminicola* Sacc. Var. *aciliolata* d' Almeida & Camara and *A. desmazieri* Cav. have been recorded on *Lolium perenne*. The description for *A. graminicola* was revised by Davis (1919 in Sprague 1950) but Sprague (1950) listed it under *A. sorghi* and the illustrations they provided showed that it was a heterogeneous group isolated from a variety of grasses. Different spore forms were identified on different grasses and it seems more likely that *A. sorghi* Sacc. *Sensu* Sprague and Johnson is an aggregate of species. The conidial state of *D. graminicola* appears distinct from that of the authenticated samples of *A sorghi.* In the current study, none of the pycnidia except for isolates three and seven were found to be rust coloured to sepia, and conidial shape was as variable as for the *Ascochyta* state of *D. exitialis* and *A. phyllachoroides* f. *melicae*. The conidia were shaped as those of the *Ascochyta* state of *D. graminicola* and not as those of *A. sorghi* and the type material of *A graminicola* var. *ciliolata*. The conidial state of *D. graminicola* has similar

measurements to *A. desmazieri* Cavara. Punithalingam (1979b) did not examine Cavara's or Saccardo's material of *A. graminicola* on *Arrhenatherum avenaceum* and the sample determined by Saccardo to be *A. graminicola* on *Lepturus incurvatus* was found upon examination to contain no *Ascochyta* spp.

The Ascochyta state of D. exitialis

The *Ascochyta* state of *D. exitialis* is distinguishable from the *Ascochyta* state of *Didymella graminicola* and *A. hordeicola* by the greater variability of the pycnidia and also on conidial morphology according to Punithalingam (1979b). In the current study it was also found to be distinct from the *Ascochyta* state of *D. graminicola* and from *A. hordeicola.* Müller (1952) stated that the conidial state of *D. exitialis* was *A.sorghi* and that *A. graminicola* (*A. graminicola* var. *diedickeana*, *A. graminicola* var. *holci*, *A. graminicola* var. *leptospora* and *A. elymi* are all synonymous with *A. sorghi).* Punithalingam disagreed as *A. graminicola* is the *Darluca filum* state of *Eudarluca caricis* and *A. sorghi* has larger conidia. *A. leptospora* has narrower conidia than those of *D. exitialis,* and *A. elymi* Tehon & Daniels is considered to be conspecific with *A. leptospora.* The other two species have an uncertain identity. Therefore *D. exitialis* cannot be referred to by any of these names and so is simply called the *Ascochyta* state.

Ascochyta phyllachoroides f. *melicae*

Ascochyta phyllachoroides f. *melicae* is associated with *A. avenae* and *A. hordei* but its conidia are morphologically different and smaller than *A. avenae* and wider and thicker walled than *A. hordei.* All the isolates of *D. phleina* in the current study had straw yellow conidia that were smaller than the published dimensions for *A. avenae* and wider than those of *A. hordei.*

Whilst there are imperfections in the methods used, there is consistency between the techniques utilised. The cross validation of the discriminant model showed that there was exceptionally good agreement between the data sets as to the species distinction. The results from the current study and the results from previous studies into graminicolous *Didymella* spp. (Punithalingam 1969 and 1979b) show that consistency

exists apart from the dimensions of the *Ascochyta* state of *D. graminicola*. The basis of the techniques used are inherently objective statistical methods although the choice of characters and the weighting of these characters, the clustering method, used and the type of similarity measure give rise to considerable subjectivity (Lam and Chapman 1985). Similar to other studies, the groupings the isolates fell into were searched by eye and hence no objective and definable indication of how large the difference between the groups should be before they can be separated into species can be definitively given. In the current study characters were not weighted, this, so as not to bias the analysis. Further the results from several analyses were combined to minimise the level of subjectivity. Few characters were examined as there was found to be no difference in the growth rates of the different isolates on PDA and CD-V_8, culture colouration was not a useful character, and neither was the shape and colouration of the ascospores and the conidia. Other criticisms of analysis such as this have suggested that reference isolates should be included. In the current study the published dimensions (Punithalingam 1969 and 1979b) of all possible species were included in the analyses and these isolates were always found within the groupings corresponding to the previously defined species.

The culture descriptions for the graminicolous *Didymella* species published by Punithalingam (1979b) do not seem to fit the culture descriptions found in this study. There was no definitive culture description for isolates of the different species and there was considerable variation in the culture descriptions within species and sometimes within isolate. This could indicate that differences in environmental conditions could play a role in the colouration of cultures, however, the majority of the cultures in this study were subjected to the same environmental conditions. More research could be directed into the pigments that graminicolous *Didymella* species produce in culture and under what conditions the colouration changes. Potentially different coloured cultures have different levels of pathogenicity (in Chapter three it was found that after mycelial discs of *Didymella* spp. were placed on wheat leaves a red colouration of the affected plant cells developed and in all cases a scorch lesion developed).

The molecular analysis was not extended any further (due to the development of a serious allergy) to encompass a broader range of isolates. The small sample investigated showed differences between 1 of the 5 isolates Further work should be carried out to establish whether the morphological analysis can be corroborated and perhaps further define the differences between the groups. The conditions for RAPD PCR are now defined for graminicolous *Didymella* spp The use of DNAzole reduces the time taken to isolate DNA and provides a comparable isolation of DNA to longer preparation methods.

In the words of Seifert *et al.* (1995) "from the perspective of the scientific method neither morphological nor sequence based taxonomic studies are inherently superior." Often DNA sequence data are considered unequivocally correct observations but reproducibility is rarely vigorously tested (Seifert *et al.* 1995).

6.0 GENERAL DISCUSSION

6.01 Introduction

This thesis aimed to gain a better understanding of the biology of *Didymella* spp. present on graminicolous hosts in New Zealand. The investigation encompassed taxonomy of isolates obtained from graminicolous hosts, the formation of the teleomorph in culture, and conditions for the development of leaf scorch symptoms on host plants. The central questions for the field investigations relate to the timing of entry of *Didymella* to a wheat crop, and effects on yield either as a pathogen or as an endophyte, and cultivar resistance.

6.02 The Taxonomy of Graminicolous *Didymella* spp.

From the use of statistical techniques such as principal components analysis, additive and hierarchical trees and discriminant analysis on the morphological data obtained, it became obvious that there are four major groupings of *Didymella* in New Zealand; *D. exitialis, D. phleina, D. graminicola* and *D. loliina.* For the isolates that both the teleomorph and anamorph measurements exist, agreement exists between the analyses as to the species identification in 12 out of the 14 isolates. The dimensions of graminicolous *Didymella* spp. obtained for all the species were within the ranges published by Punithalingam (1979b) except for the *Ascochyta* state of *D. graminicola.* The pycnidial diameter and the length and the width of the conidia were larger than those reported by Punithalingam (1979b) but where the anamorph state and the teleomorph state existed for the *D. graminicola* isolates, both states were identified as *D. graminicola.* Two *Ascochyta* states of *D. graminicola* could also be identified as *A. sorghi.* Application of molecular techniques would be useful in helping to resolve this complex situation.

No investigation had previously been carried out into the molecular characterisation of *Didymella* spp. Clamped homogeneous electric field (CHEF), Restriction amplified polymorphic DNA (RAPD), and finally restriction digests of the

RADP fragment were used in an attempt to provide a clearer picture of the taxonomy of *Didymella* spp. Conditions for this work have been defined and the results obtained indicate the presence of two species amongst the five isolates examined. The two species identified were *D. exitialis* (4 isolates) and *D. phleina* (1 isolate). The identifications achieved using the molecular work agreed with the species identification achieved using the morphological taxonomy.

With respect to sporulation, some fungi appear to be relatively indifferent and independent of their environment while others appear to require a unique combination of environmental conditions (Lilly and Barnett 1951). *Didymella* appears to need a unique, as yet undefined, combination of environmental conditions for teleomorph development within the laboratory. Sexual reproduction of many fungi occurs only as the vegetative growth is near or past the maximum. Many of the cells of the mycelium are at this time dead or dying and autolysis follows and the remaining alive cells absorb their concentration of essential substances to such a degree that reproduction is possible (Lilly and Barnett 1951). Some cultures of *Didymella* that were drying down produced the teleomorph, indicating that, in the case of *Didymella,* cell death may be a trigger for sporulation.

The production of perithecia of *Ophiobolus graminis* depended on the carbon and nitrogen ratio of any particular medium (Weste and Thrower 1963) but differences in nitrogen had little or no effect on *Didymella* in culture. Higher levels of glucose or other carbon sources increased the amount of aerial mycelia (Barnett and Lilly 1947b in Capral 1958, Hawker 1951). Mead (1962) found that pycnidial development of *Ascochyta imperfecta* Peck. depended on nitrogen being added to the medium but not the type of nitrogen. Similarly in this study, nitrogen was required for production of pycnidia of *Didymella*, but the type of nitrogen salt did not matter.

Other fungi, for example *Sclerotium rolfsii*, infrequently produce their teleomorph either in nature or the laboratory, but the teleomorph (of *Sclerotium rolfsii*) was consistently produced using corn leaf culture medium (Tu *et al.* 1992). Some

Ascochyta spp., for example *Ascochyta rabiei,* require artificially infested chickpea straw and high moisture and low temperatures for the induction of their teleomorphs. On normal and low nutrient media pseudothecia failed to grow therefore it is thought that some nutrients or factor from the chickpea straw encourages sporulation (Trapero-Casas and Kaiser 1992). In this study media containing plant parts did not induce the formation of the *Didymella* state in culture. Buston *et al.* (1953 in Campbell 1958) found that calcium was essential for perithecial formation of several species of *Chaetomium* but the addition of calcium to media *Didymella* was grown on did not induce the teleomorph to form.

The longer duration of green leaf area found by Cromey and Mace (1995) does not appear to be associated with the control of *Didymella* as the fungicides used did not control *Didymella* effectively during the 1996/97 wheat growing season. The extra days of green leaves appear to be due to either the hormone like effects of the fungicides or the control of other fungal pathogens or endophytes. Similar results were found by Riesen and Close (1987), where foliar application of propiconazole resulted in higher barley yields up to 11% greater than for the untreated control.

In both years investigated, *Didymella* spp. were cultured readily from symptomless green leaf leaves. This agrees with other researchers (Ahrens and Schöpfer 1983, Cromey *et al.* 1994), and suggests that *D. exitialis* probably exists endophytically within leaf tissue. The fungus was present in all four trials during the 1995/96 season, although its frequency was highest following the application of tebuconazole. During the 1996/97 season there was no difference between the amounts of *Didymella* recorded in the control and the two fungicide treatments. Retardation of senescence was obtained in fungicide treated plots, but whether this was due to the control of saprophytic fungi or hormone-like effects of the fungicides remains unknown.

Newly unfolded leaves of annuals, for example wheat, are predominantly sterile and the resultant habitat is of a transient nature existing at most for a few months. Exponential growth of micro-organisms such as yeasts and bacteria in the phyllosphere

develop until a steady state is reached when limitation of nutrients stops any further growth (Fokkema and Schippers 1986 and Fokkema 1988). This was shown in the numbers of bacteria, yeast and fungi isolated from wheat leaves in the present study. Very few bacteria were isolated after the leaves had initially unfolded and the numbers of organisms in the phylloplane increased until just before leaf senescence when sampling stopped.

Riesen (1985 in Riesen 1987) and Sieber (1985 in Riesen 1987) demonstrated that known pathogens of wheat are present throughout the vegetative period of growth. Known pathogens were isolated only on rare occasions in the current study. *S. tritici* was never isolated from wheat leaves. *Fusarium* spp., *S. nodorum* and *Helminthosporium* sp. were the only pathogens isolated during this study. Their presence was transient and when they were present they could only be cultured from, on average, five percent of leaves.

Only ten species of fungi were isolated from the wheat leaf laminar, fewer than the 14 species that Riesen (1987) isolated from barley leaves. The weather conditions in the seasons examined during this study were relatively dry and cool but there was no correlation between weather conditions and the levels of *Didymella* and *Sporobolomyces* within the leaf laminar. A combination of environmental conditions may affect the levels of *Didymella*. The number of endophytes isolated decreased when there was little precipitation and also as the area of green leaf tissue decreased (Riesen and Close 1988). Sieber *et al.* (1988) isolated over 200 spp. of fungi from wheat leaves. The difference between the results of this current study and the study of Riesen (1987), compared to the work of Sieber *et al.* (1988) in Switzerland, may be due to the geographic isolation of New Zealand, the hot dry windy conditions of the Canterbury Plains, or a combination of the two.

Only a small number of the organisms present on aerial plant parts are pathogens, the majority are saprophytes living on dead organic material (Smedgaard-Petersen and Tolstrup 1985). *Cladosporium* spp., *Aureobasidium pullans, Alternaria*

spp., *Sporobolomyces* spp. (pink yeasts), *Cryptococcus* spp. (white yeasts) account for the majority of the fungi present (Fokkema 1971, Dickinson 1973, Diem 1974, Flannigan and Campbell 1977, Tolstrup and Smedgaard-Petersen 1984 in Jachmann and Fehrman 1989).

Numerous studies have shown in both wheat and barley (Griffiths and Scott 1977 in Griffiths 1981) that the application of fungicides increases yield even if disease levels are zero or very low. On the other hand several researchers have suggested that the saprophytes themselves are having a detrimental effect on yield (Dickinson and Walpole 1975, Mappes and Hampel 1977, Cook 1980, Priestly and Bayles 1982).

Saprophytes can cause a reduction in yield by the depletion of a plant's stored energy, which thus causes a reduction in plant growth (Dickinson 1973, Diem 1974, Smedgaard-Petersen and Stølen 1981, Smedgaard-Petersen and Tolstrup 1985). Pathogens which are not sensitive, or pathogens that have become resistant, to the fungicides applied will certainly profit from the reduction of saprophytic mycoflora (Hislop 1976). Reported increases in the field of *Alternaria* and *Cochliobolus* diseases mainly on other crops after treatment with benzimidazole fungicides may be explained in this way. However, clear evidence about the role of the saprophytes in the field is mostly lacking. In the present study, saprophytic fungi remained at similar levels to those pre fungicide application levels indicating that saprophytes played a minor role in hastening leaf senescence and thus a minor role in reducing wheat yield. It would be difficult to obtain information about the role of saprophytes in leaf senescence because of changing environmental conditions and the interaction effects between saprophytic and pathogenic fungi.

Magan and Lacey (1986) studied the colonisation of wheat by various micro-organisms using two wheat cultivars Timmo (1980) and Maris Huntsman (1981-82). The level of micro-organisms increased between anthesis and harvest on flag leaves and ears. Yeasts, yeast like fungi and filamentous fungi, predominated but varied as to their actual individual abundance. Yields increased by 2.4% after a single

fungicide spray at GS50 or 60, when another spray time at GS 38-40 was added the increase was 5.1%. An increase in grain weight accounted for most of the yield gain. In the present study the yield increased after a single application of fungicide with no differences between the timing of fungicide application. Most of the yield increase was accounted for by increases in grain weight.

Didymella appears, from the results gained during this study, to be an either an endophyte sporulating on senesced material or a latent pathogen which does not cause severe symptoms under the environmental conditions experienced in the seasons investigated. It penetrates the host directly and then exists in a subcuticular intramural position. The optimal conditions for the production of symptoms were not elucidated in this study. If the period before symptom development on artificially inoculated plants was to be classified as a true latent period, *Didymella* spp. would have to eventually induce macroscopic symptoms and sporulate within the lesion (Zadoks 1978). In *Didymella*'s case it does produce symptoms in glasshouse experiments, although inconsistently, affecting at most one percent of the leaf area. However, to sporulate, it requires exposure to near -UV light. In the field scorch symptoms are produced rarely and sporulation of the fungus on leaf material is uncommon until later in the field season, when leaves senesce.

In soybean (*Glycine max* L.), plants in the field have been observed with signs or symptoms of disease, but which were in fact killed by abiotic factors (Sinclair 1991). In soybean, the effects of pathogens in their latent phase on the plant are unknown; some may have latent periods of up to several weeks. The latent period may predispose plants to other diseases, or alternatively weaken it but it also reduces the potential inoculum (as the pathogens are latent they are not producing inoculum). The fungus that produces the most obvious symptoms is often thought to have caused plant death but the pathogen may have become active only when the host plant became stressed.

This is perhaps what is happening in the case of *Didymella* spp. in New Zealand. *Didymella* enters the plant and survives in a latent state until plant senescence or abiotic

stresses cause premature leaf senescence. It then sporulates profusely on the dead leaf tissue which leads to the assumption that it played a major role in leaf senescence. The yield data obtained suggests that *Didymella* spp. are at most weak pathogens but because of its resistance to fungicides, this was not be confirmed. Yield increases, although not as large as azoxystrobin treated plots, were obtained in tebuconazole treated plots where the level of *Didymella* sporulation was higher than the untreated control in some cases. Fungicide treatment, as has been shown in other studies to increase the yield of wheat crops, but the actual reasons for this increase remain unclear.

Initially after the yield increase was noted during the 1995/96 growing season, and the level of sporulation of *Didymella* had increased in the tebuconazole treated plots, the possibility of another organism controlling *Didymella* levels was suggested (the levels of *Sporobolomyces* were investigated as were other phyllosphere organisms, and within the leaf tissue pre-fungicide levels of these organisms were returned to within a short period of time). The lack of control of *Didymella* was noted also in laboratory based tests, with tebuconazole only reducing mycelial growth and azoxystrobin only reducing germination of conidia.

The economic importance of diseases caused by *D. phleina* and *D. exitialis* does not appear to be great in other countries. *D. phleina* has been recorded as a pathogen of *Phleum pratense* L. and other grasses in Norway under favourable conditions, but the term favourable conditions was not defined (Årsvoll 1975 in Riesen 1987). There is little information available about the epidemiology of graminicolous *Didymella* spp., however there is evidence that they occur in leaves of graminicolous host as endophytic fungi. *A. paspali* (Sydow) Punith. was isolated from *Paspalum dilatatum* Poir and its systemic growth in the xylem was also shown (Buchanan 1984). Riesen (1985 in Riesen 1987) and Sieber (1985 in Riesen 1987) isolated both *D. exitialis* and *A. hordei* var. *europa* Punith. from green wheat leaves.

Overall, it appears that *Didymella* is either an endophyte or a weak pathogen. This confirms the results of Ahrens and Schöpfer (1983). It does not appear to play a

major role in yield reduction and therefore currently no resistant cultivars or fungicides are required for its control. Further monitoring under a broader range of environmental conditions, for example, a comparatively wet growing season or a dry season would provide more information about the levels of *Didymella* and whether or not it can cause greater than 1% leaf scorching. Taxonomically it appears that we do have *D. graminicola, D. phleina, D. exitialis* and *D. loliina* present in New Zealand on the crops and grasses surveyed in this study.

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8.0 APPENDIX I – BUFFERS USED

8.01 Citrate Phosphate buffer

Stock solution: 0.1 *M* citric acid (19.21 g/l) and 0.2 *M* Na_2HPO_4 (53.65 g/l). Mix *x* ml of citric acid to *y* ml of phosphate solution and dilute the mixture to one litre. For pH 4.8: use 252 ml of citric acid, and 248 ml of Na_2HPO_4.

8.02 Phosphate buffer

Stock solution: 0.2 *M* NaH_2PO_4 (31.2 g/l) and 0.2 *M* $Na_2HPO_4 \cdot 7H_2O$ (53.65 g/l). Mix *x* ml of NaH_2PO_4 solution to *y* ml of $Na_2HPO_4 \cdot 7H_2O$ and dilute to one litre. For pH 5.8: use 460 ml of NaH_2PO_4 and 40 ml of $Na_2HPO_4 \cdot 7H_2O$.

8.03 Glycine – NaOH buffer

Stock solution: 0.2 *M* glycine (15.01 g/l) and 0.2 *M* NaOH. To 50 ml of glycine add *x* ml of NaOH. For pH 8.6: use 4 ml of NaOH.

9.0 APPENDIX II – MARTIN'S MEDIUM (Dhingra and Sinclair 1995)

Martins medium

Yeast extract	0.5g
Dextrose	10g
Peptone	5g
Agar	15g
KH_2PO_4	1g
$MgSO_4.7H_20$	0.5g
Rose bengal	0.05g
KH_2PO_4	0.5g

Adjust to pH 5.

10.0 APPENDIX III – LIST OF ISOLATES AND IDENTIFIERS FOR TELEOMORPH STATE

Isolate	Species	Pseudothecia Diameter	Ostiole diameter	Ascus length	Ascus width	Ascospore length	Ascospore width	Year
1	1	100.1	23.9	39.0	8.6	18.0	5.9	96/97
2	1	88.5	20.7	41.9	7.9	16.4	5.9	96/97
3	1	104.7	19.5	45.2	9.9	15.9	4.0	96/97
4	1	103.4	22.0	41.0	9.9	13.0	4.0	96/97
5	1	95.6	21.3	44.5	9.9	14.7	4.0	96/97
6	1	102.5	19.8	45.4	9.9	15.8	4.0	96/97
7	1	106.0		39.6	9.9	16.2	4.0	96/97
8	1	90.6	19.8	41.9	9.9	14.6	4.0	96/97
9	1	115.8	22.6	43.1	9.9	15.2	4.0	96/97
10	1	87.6	19.8	43.6	9.9	13.9	4.0	96/97
11	1	109.3	25.3	42.0	9.9	15.4	4.0	96/97
12	1	96.6	23.9	43.3	9.9	15.4	4.0	96/97
13	1	100.2	27.7	39.6	9.9	15.8	4.0	96/97
14	1	74.6						96/97
15	1	99.1		44.8	9.9	15.9	4.0	96/97
16	1	77.8	28.4	49.5	9.9	14.5	4.0	96/97
17	1	85.7		42.0	9.9	15.5	4.0	96/97
18	1	97.7		40.9	9.9	16.7	4.0	96/97
19	1	109.6	24.8	42.6	9.9	14.9	4.0	96/97
20	1	114.8	23.8	39.6	9.9	15.8	4.0	96/97
21	1	86.1						96/97
22	1	100.4	20.2	43.6	9.9	15.2	4.0	96/97
23	1	100.2		39.6	9.9	13.9	4.0	96/97
24	1	103.0	20.9	44.1	9.9	14.7	4.0	96/97
25	1	97.8		37.4	9.9	14.8	4.0	96/97
26	1	94.0	29.7	40.2	9.9	15.4	4.0	96/97
27	1	94.5	29.7	41.0	9.9	14.4	4.0	96/97
28	1	84.2	21.3					96/97
29	1	93.9	19.8	40.4	9.9	13.9	4.0	96/97
30	1	98.3		42.8	9.9	13.5	4.0	96/97
31	1	92.6		37.8	9.9	15.4	4.0	96/97
32	1	109.0		42.2	9.9	16.5	4.2	96/97
33	1	100.2		49.5	9.9	15.2	4.0	96/97
34	1	119.0	26.5	42.6	9.9	14.5	4.0	96/97
35	1	94.9	22.5	42.3	9.9	15.5	4.0	96/97
36	1	110.9		44.8	9.9	13.9	4.0	96/97
37	1	96.4	24.9	42.1	9.9	13.5	4.0	96/97
38	1	103.3		49.1	9.9	13.9	4.0	96/97
39	1	95.9		41.8	9.9	14.3	4.0	96/97
40	2	114.8	27.7	50.7	9.9	15.8	4.0	96/97
41	1	94.0						96/97
42	1	99.8		39.6	9.9	14.3	4.0	96/97
43	1	98.8	17.8	39.6	9.9	15.2	4.0	96/97
44	1	90.9		42.2	9.9	15.8	4.0	96/97
45	1	111.4		41.6	9.9	14.7	4.0	96/97
46	2	132.9		64.0	11.0	19.3	5.5	95/96
47	2	143.4		57.7	11.0	19.3	5.5	95/96
48	1	115.6		41.9	11.0	16.0	5.1	95/96
49	1	107.4		46.3	11.0	14.6	5.4	95/96

Teleomorph state Cntd.

Isolate	Species	Pseudothecia Diameter	Ostiole diameter	Ascus length	Ascus width	Ascospore length	Ascospore width	Year
50	**2**	115.9		62.6	11.0	17.9	5.5	**95/96**
51	**1**	109.2		49.6	8.4	12.6	4.1	**95/96**
52	**2**	139.5	33.5	57.4	11.0	21.8	5.5	**95/96**
53	**2**	130.8		59.0	11.0	19.1	5.5	**95/96**
54	**1**	138.1		47.7	9.2	15.4	4.3	**95/96**
55	**2**	105.0		50.2	9.4	16.6	4.4	**93/94**
56	**1**	115.2		39.2	8.6	12.9	3.0	**93/94**
57	**1**	120.5		49.4	10.5	15.1	4.1	**93/94**
58	**2**	136.1		47.6	10.8	18.1	4.9	**93/94**
59	**2**	143.3		45.3	8.7	15.4	4.5	**93/94**
60	**2**	128.7		49.9	9.9	13.1	3.4	**93/94**
61	**2**	99.2		36.6	6.2			**93/94**
62	**1**	122.1		53.1	9.7	16.1	4.9	**93/94**
63	**2**	127.1		52.2	11.2	17.1	4.9	**93/94**
64	**2**	131.0		52.2	8.2	13.1	4.9	**93/94**
65	**2**	140.3		53.8	8.2	16.6	4.8	**93/94**
66	**2**	126.4		50.7	8.5	15.4	4.9	**93/94**
67	**2**	123.8		45.5	9.1	15.8	4.0	**93/94**
68	**2**	126.7		45.5	9.9	15.1	4.0	**93/94**
69	**2**	122.8		50.4	10.1	16.4	4.2	**93/94**
70	**2**	104.9		47.9	9.9	15.6	4.0	**93/94**
71	**2**	130.7		45.3	9.9	15.6	4.0	**93/94**
72	**2**	135.0		46.9	9.9	16.8	4.0	**93/94**
73	**2**	133.7		46.9	9.9	14.7	4.0	**93/94**
74	**2**	121.0		49.7	9.9	11.9	4.0	**93/94**
75	**2**	138.6		50.9	9.3	16.4	4.0	**93/94**
76	**2**	143.2		46.8	9.9	14.9	4.0	**93/94**
77	**1**			43.9	8.7	14.9	3.7	**93/94**
78	**1**			47.7	9.9	16.1	4.0	**93/94**
79	**2**	140.3		50.5	9.9	16.7	4.0	**93/94**
80	**1**	114.2		44.6	9.9	15.9	4.0	**93/94**
81	**1**	124.7		46.9	9.9	15.7	4.0	**93/94**
82	**2**	128.0		45.3	9.9	15.4	4.0	**93/94**
83	**4**	158.4		47.1	9.9	15.2	4.0	**93/94**
84	**1**	93.5		43.8	10.5	16.6	3.9	**93/94**
85	**1**	91.6		44.9	10.9	16.7	4.5	**93/94**
86	**1**	107.4		50.4	13.6	16.9	4.9	**93/94**
87	**1**	96.2		50.0	8.2	15.7	4.9	**93/94**
88	**2**	134.6		48.9	12.3	16.8	4.0	**93/94**
89	**2**	117.2		47.7	9.7	16.6	4.0	**93/94**
90	**2**	124.7		44.0	9.3	14.5	4.0	**93/94**
91	**1**							**93/94**
92	**1**			47.9	8.4	12.5	3.4	**93/94**
93	**1**	112.8		38.2	5.8	10.3	1.8	**93/94**
94	**1**			47.3	9.3	15.0	4.6	**93/94**
95	**1**	111.3		42.3	9.4	14.8	3.8	**93/94**
96	**2**	130.5		37.6	8.9	15.6	3.9	**93/94**
97	**2**	120.1		46.1	8.4	16.3	4.4	**93/94**
98	**2**	141.1		41.0	9.2	14.1	3.8	**93/94**
99	**1**	106.6		44.8	8.2	13.6	4.3	**93/94**
100	**2**	116.6		50.2	10.0	18.5	4.3	**93/94**
101	**1**	105.1		48.2	9.9	16.6	4.0	**93/94**

Teleomorph state Cntd.

Isolate	Species	Pseudothecia Diameter	Ostiole diameter	Ascus length	Ascus width	Ascospore length	Ascospore width	Year
102	**1**	110.2		41.8	11.3	14.8	5.0	**93/94**
103	**1**	110.9		40.6	7.9	17.2	4.0	**93/94**
104	**1**	114.6		43.2	7.1	13.1	4.9	**93/94**
105	**2**	123.0		43.3	9.9	15.1	4.0	**93/94**
106	**2**	121.9		50.5	7.9	15.8	4.0	**93/94**
107	**2**	130.7		42.7	9.3	15.8	4.0	**93/94**
108	**2**	130.7		42.7	9.3	15.8	4.0	**94/95**
109	**1**	110.0		50.0	10.0	13.0	4.8	**94/95**
110	**2**	130.0		60.0	11.0	16.5	4.5	**94/95**
111	**4**	180.0		45.0	11.0	16.5	3.5	**94/95**
112	**3**	220.0		55.0	12.5	13.0	4.3	**94/95**
113	**1**	109.6		49.7	9.9	14.5	4.0	**94/95**
114	**1**	108.5		48.9	9.9	19.4	4.0	**94/95**
115	**2**	118.6		49.1	9.9	15.8	4.0	**94/95**
116	**1**	112.9		47.0	10.0	15.6	4.0	**94/95**
117	**2**	120.1		47.6	9.9	15.0	4.0	**94/95**
118	**2**	120.2		45.3	9.7	14.9	4.0	**94/95**
119	**1**	98.2		38.1	8.2	13.1	2.7	**95/96**
120	**1**	111.5		46.7	9.2	13.9	4.0	**95/96**
121	**1**	111.8		44.9	9.9	15.5	4.0	**95/96**
123	**1**	96.1		40.9	9.9	12.3	4.0	**95/96**
124	**1**	111.2		49.4	10.7	17.4	2.8	**95/96**
125	**2**	132.9		64	11	19.3	5.5	**95/96**
126	**2**	143.4		57.7	11	19.3	5.5	**95/96**
127	**1**	115.6		41.9	11	16	5.1	**95/96**
128	**2**	115.7		62.6	11	17.9	5.5	**95/96**
129	**1**	109.2		49.6	8.4	12.6	4.3	**95/96**
130	**2**	139.5		57.4	11	21.8	5.6	**95/96**
131	**2**	130.8		59	11	19	5.6	**95/96**
132	**1**	105		50	10	15	3.8	**95/96**
133	**1**	87.5		43.6	9.9	11.9	4	**95/96**
134	**1**	104.9		60.72	9.1	16.6	2.8	**95/96**
135	**1**	98.9		46.5	9.9	13.5	4	**95/96**
136	**1**	100.8		42.6	10	19.1	4	**95/96**
137	**1**	118.7		49	9.9	15.8	4	**95/96**
138	**1**	112.9		47	9.9	15.6	4	**95/96**
139	**1**	120.1		47.7	9.9	13.1	4	**95/96**
140	**1**	120.2		45.3	9.7	14.9	4	**95/96**
141	**1**	98.2		38.1	8.2	13.1	4	**95/96**
142	**1**	111		46.7	9.2	13.9	4	**95/96**
143	**1**	111.7		44.9	9.9	15.5	4	**95/96**
144	**1**	96.1		40.9	9.9	12.3	4	**95/96**
145	**1**	111		50	10.7	17.4	2.8	**95/96**

11.0 APPENDIX IV – LIST OF ISOLATES AND IDENTIFIERS FOR ANAMORPH STATE

Isolate no	Species identification	Pycnidial diameter	Condial length	Condial width	Year
1	1	99.0	18.4	5.9	96/97
2	4	242.2	18.1	5.9	96/97
3	4	196.2	18.2	5.9	96/97
4	4	225.4	19.3	5.9	96/97
5	4	215.9	18.0	5.9	96/97
6	4	214.5	19.6	5.9	96/97
7	4	199.9	16.1	5.9	96/97
8	1	142.6	17.6	4.3	96/97
9	1	146.5	17.6	5.2	96/97
10	4	199.1	17.0	4.0	96/97
11	2	193.6	18.3	5.3	96/97
12	1	133.9	18.2	4.0	96/97
13	1	113.3	19.2	5.6	96/97
14	1	125.2	18.8	5.9	96/97
15	1	128.9	17.7	5.9	96/97
16	1	136.7	18.7	5.9	96/97
17	1	119.9	14.0	5.9	96/97
18	1	126.2	18.7	4.0	96/97
19	1	126.8	17.5	6.0	96/97
20	1	117.8	17.7	5.9	96/97
21	1	120.1	17.2	5.9	96/97
22	2	165.0	16.6	5.9	96/97
23	1	145.3	17.5	5.9	96/97
24	1	144.1	17.3	5.9	96/97
25	1	110.6	19.2	5.9	96/97
26	1	133.8	16.7	5.9	96/97
27	1	133.6	16.8	4.0	96/97
28	2	170.6	19.3	6.0	96/97
29	2	179.8	19.4	4.0	96/97
30	2	160.5	21.0	5.9	96/97
31	2	158.0	20.6	5.9	96/97
32	1	112.4	18.2	3.8	93/94
33	2	191.4	24.3	5.5	93/94
34	1	121.4	14.5	3.8	93/94
35	2	182.5	18.8	5.0	93/94
36	1	113.0	15.0	3.8	93/94
37	1	137.6	16.9	5.0	93/94
38	4	260.2	19.2	5.6	93/94
39	2	182.0	18.7	4.6	93/94
40	1	142.0	18.0	4.9	93/94
41	1	119.3	17.3	5.5	93/94
42	1	108.2			93/94
43	1	142.6	15.4	3.2	93/94
44	1	135.3	15.1	3.4	93/94
45	2	179.8	13.9	4.0	93/94
46	2	195.9	18.4	3.8	93/94
47	4	211.9	15.6	3.3	93/94
48	4	208.3	17.2	3.3	93/94
49	4	234.7	17.0	3.4	93/94

Anamorph State Cntd.

Isolate no	Species identification	Pycnidial diameter	Condial length	Condial width	Year
50	1	125.4	15.3	3.3	93/94
51	1	118.8	15.6	3.3	93/94
52	2	156.9	16.0	3.7	93/94
53	4	234.7	16.2	4.0	93/94
54	2	177.1	14.2	3.3	93/94
55	1	120.0	14.3	3.8	93/94
56	2	181.0	15.5	4.0	93/94
57	1	128.7	15.8	4.0	93/94
58	1	119.5	17.8	4.0	93/94
59	1	120.1	17.6	4.1	93/94
60	1	104.2	14.9	4.0	93/94
61	2	189.5	17.2	4.4	93/94
62	2	160.0	16.8	4.0	93/94
63	1	121.8	18.1	5.3	93/94
64	2	169.3	16.2	4.9	93/94
65	1	120.7	14.5	3.6	93/94
66	1	147.4	14.7	3.3	93/94
67	1	122.8	14.5	4.0	93/94
68	2	177.5	17.8	4.5	93/94
69	1	136.7	16.6	4.0	93/94
70	1	122.8	17.0	4.3	93/94
71	2	156.3	15.6	3.7	93/94
72	1	119.6	13.9	4.0	93/94
73	4	225.6	14.9	4.9	93/94
74	1	123.0	16.6	4.0	93/94
75	1	140.5	17.2	4.0	93/94
76	2	184.6	13.9	4.7	93/94
77	4	197.7	15.3	4.6	93/94
78	1	114.8	14.6	4.1	93/94
79	1	140.2	14.8	4.0	93/94
80	1	146.3	18.4	4.2	93/94
81	1	141.0	16.2	4.0	93/94
82	2	153.3	16.8	4.5	93/94
83	3	133.9	12.7	4.1	93/94
84	1	136.3	14.3	4.1	93/94
85	1	115.6	16.2	4.1	93/94
86	1	132.2	14.8	3.7	93/94
87	3	99.3	8.2	3.3	93/94
88	1	113.6	15.4	4.6	93/94
89	2	158.9	16.6	4.2	93/94
90	1	131.7	14.9	4.3	93/94
91	2	173.8	16.1	4.0	93/94
92	1	120.0	16.5	3.2	94/95
93	1	134.8	18.1	4.9	94/95
94	1	108.4	16.8	4.9	94/95
95	1	108.1	16.4	4.0	94/95
96	1	149.9	14.1	3.8	94/95
97	1	102.6	13.9	4.0	94/95
98	2	157.1	14.1	4.0	94/95
99	1	119.0	13.4	4.0	94/95
100	2	164.4	17.0	5.2	94/95

Anamorph State Cntd.

Isolate no	Species identification	Pycnidial diameter	Condial length	Condial width	Year
101	**1**	138.4	18.9	5.3	**94/95**
102	**2**	170.4	20.7	5.3	**94/95**
103	**1**	147.0	15.5	4.1	**94/95**
104	**2**	174.0	19.0	5.9	**94/95**
105	**4**	225.0	18.3	4.0	**94/95**
106	**1**	138.7	19.0	4.0	**94/95**
107	**1**	138.2	19.2	5.9	**94/95**
108	**1**	107.9	15.6	4.3	**94/95**
109	**2**	188.9	15.7	4.1	**94/95**
110	**4**	196	24.1	5.4	**94/95**
111	**1**	135.9	16.6	3.8	**94/95**
112	**1**	137.5	15.9	4.1	**94/95**
113	**1**	124.1	17.0	4.2	**94/95**
114	**1**	147.1	16.9	3.6	**94/95**
115	**2**	154.0	18.4	5.0	**94/95**
116	**1**	142.0	16.7	5.3	**92/93**
117	**2**	174.0	16.7	4.8	**92/93**
118	**1**	139.0	15.5	4.7	**92/93**
119	**2**	157.0	18.8	5.6	**92/93**
120	**2**	191.0	17.6	5.0	**92/93**
121	**2**	178.0	18.3	5.5	**92/93**
122	**1**	112.4	18.5	3.75	**94/95**
123	**1**	130.1	16	5	**94/95**
124	**4**	191.1	24.3	5.6	**94/95**
125	**1**	121.5	14.5	3.8	**94/95**
126	**2**	182.5	18.8	5	**94/95**
127	**1**	113	15	3.8	**94/95**
128	**1**	137.6	16.9	5	**94/95**
129	**4**	260	19.2	5.6	**94/95**
130	**1**	110	18.5	3.8	**95/96**
131	**4**	200	24.3	5.5	**95/96**
132	**1**	121.4	15	3.8	**95/96**
133	**2**	182.5	18.8	5	**95/96**
134	**1**	113	15	3.8	**95/96**
135	**4**	260.2	19.2	5.6	**95/96**
136	**1**	130	17.5	5	**95/96**

12.0 APPENDIX V - ISOLATES OF GRAMINICOLOUS *DIDYMELLA* SPP. USED IN CHAPTER 2.0: FIELD INVESTIGATIONS OF *DIDYMELLA* SPP.

Refer to Appendices III and IV for more information on the isolates.

12.01 The effects of tebuconazole and azoxystrobin on graminicolous *Didymella* spp.

Isolate: 130, 131, 132, 133.

13.0 APPENDIX VI – ISOLATES OF GRAMINICOLOUS *DIDYMELLA* SPP. USED IN CHAPTER 3.0: INFECTION PROCESSES OF *DIDYMELLA* SPECIES

Refer to Appendices III and IV for more information on the isolates.

13.01 Preparation of spore suspensions

Isolates: 50, 51, 52, 53, 54, 55.

13.02 Optimisation of infection conditions

Same isolates as used for **13.01**.

13.03 Comparsion of infection processes on attached versus detached leaves and inoculum effects

Same isolates as used for **13.01.**

13.04 The effects of humidity and temperature on spore germination and appressorial formation

Isolates: 1, 8

14.0 APPENDIX VII – ISOLATES OF GRAMINICOLOUS *DIDYMELLA* SPP. USED IN CHAPTER 4.0: *IN VITRO* INVESTIGATIONS OF *DIDYMELLA* SPP.

Refer to Appendices III and IV for more information on the isolates.

14.01 Growth rates

Isolates: 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 80, 81, 82, 83, 84, 85.

14.02 Carbon and Nitrogen sources and the effects of pH on the radial growth of graminicolous *Didymella* in culture

Isolates (94/95): 92, 93, 94, 95, 96, 97, 98, 99, 100, 101.

(95/96): 130, 131, 132, 133, 134, 135, 136.

(96/97): 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

14.03 Mating of isolates

Isolates: 130, 131, 132, 133, 134, 1, 2, 3, 4, 5.

14.04 Different inoculum sources and age of inoculum

Isolates: 1, 2, 3, 4, 5.

14.05 The effects of plant material on sporulation

Isolates: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

14.06 Effects of temperature on the growth of *Didymella* in culture

Isolates: 130, 131, 132, 133, 134, 135, 136, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

14.07 The use of paraquat

Isolates: 1, 2, 3.